
Effect of Thaw Temperature on Murine Blastocyst Development

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Received June 5, 2009

Assisted Reproductive Technology (ART) methods are employed to help infertile couples conceive. One such ART procedure is in vitro fertilization (IVF) and embryo culture (EC). During IVF-EC, supernumerary embryos are often produced and cryopreserved for future use. Embryos frozen and thawed have reduced rates of blastocyst development. Thawing temperature is one of the factors thought to affect embryo development after cryopreservation. The objective of this study was to determine if varying thaw temperatures affect embryo development. Using a randomized, controlled study, approximately 230 two-cell murine embryos were exposed to cryoprotectants and cryopreserved in lots of 15 embryos per straw. A total of 16 straws were cryopreserved. Cryopreserved embryos were thawed in water baths set at either 30°C (n = eight straws) or 37°C (n = eight straws). Once the straws were thawed and the cryoprotectant removed, the embryos were incubated for 72 hours and assessed for blastocyst development. Proportions between blastocyst developments were analyzed using the Chi square test. Blastocyst development for straws thawed at 30°C was 52.1% (63/121) compared to 45.5% (50/110) for straws thawed at 37°C. These values were not significantly different ($P = 0.4$). In conclusion, the range of thaw temperature used for this study had no effect on post-thaw embryo development. Future studies should assess other aspects of the thawing procedure, including length of time straws remain in the water bath, as well as the effect of pre-thawing straws at room temperature before placing them in the water bath.

Introduction

For three decades, Assisted Reproductive Technology (ART) has been helping infertile couples conceive. Since the birth of the first “test-tube baby” in 1978, reproductive technology has made many advances in its ability to overcome infertility (Sher et al., 2005). Couples are classified “infertile” if they have not used contraception for a year or more and have not conceived. In 2004, nearly 50,000 infants were born in the United States to “infertile” couples because of ART (Centers for Disease Control and Prevention, 2006).

An in vitro fertilization (IVF) and embryo culture (EC) cycle begins when a woman starts taking fertility drugs or has her ovaries monitored for follicular development (Wright et al., 2004; Centers for Disease Control and Prevention, 2006). The next step is egg-retrieval, after which the eggs (oocytes) are combined with sperm in the laboratory. The subsequent embryos are evaluated morphologically and two to three of the “best” embryos are transferred to the patient. If the transfer is successful and implantation occurs, the cycle is deemed a clinical pregnancy. The last step, and ultimate goal, is a live-birth delivery, defined as a birth resulting in one or more live born neonates (American Society for Reproductive Medicine, 2004). The Center for Disease Control and Prevention reported that of the 127,977 IVF-EC cycles initiated in 2004, 36,760 (28.7%) resulted in live-birth deliveries.

In many cases, infertile women have to undergo multiple cycles before pregnancy is established, and even more cycles before a live-birth is obtained. Often times, with more cycles initiated comes an increase in number of

embryos placed back into the uterus. This increased number of embryos transferred often translates into a multiple pregnancy. Unfortunately, multiple pregnancy often leads to serious consequences, especially if the pregnancy results in triplet births or greater. Therefore, multiple-infant pregnancies should be considered a complication of ART, as they are associated with a number of problems including higher rates of caesarean section, prematurity, low birth weight, and infant disability or death. Nearly one-third of pregnancies resulting from ART conclude in multiple-infant live births.

Distributing the embryos obtained during the course of one IVF-EC cycle over a number of cycles is a beneficial alternative to undergoing fresh retrievals each time (Edgar et al., 2000). Cryopreserving the embryos from the first cycle and using them for later cycles fully uses the potential of the first oocyte retrieval, while allowing all the embryos resulting from that cycle to be used rather than discarded (Jones et al., 1997; Schnorr et al., 2000; Edgar et al., 2005). Cryopreserving also provides a useful tool for avoiding multiple-infant pregnancies. Instead of transferring numerous embryos in the first transfer, some may be stored for another transfer, thus reducing the risk of multiple infants (Oehninger et al., 2000; Cohen et al., 2001) and increasing the probability of obtaining a pregnancy with one ART cycle (Jones et al., 1997).

Another complication with the IVF-EC cycle is ovarian hyperstimulation syndrome (OHSS), and occurs in an estimated 1% to 10% of ART cases when the ovaries are stimulated for ovulation induction (Fasouliotis and Schenker, 2005). In extreme cases, OHSS can cause severe

morbidity and even death. If the woman fails to conceive, the symptoms disappear rapidly; however, if a pregnancy is obtained, the symptoms may become progressively worse (Sher et al., 2005). According to Chung et al. (2006) and Edgar et al. (2005), OHSS is a greater risk in multiple-infant pregnancies and because of this, it is recommended that patients with severe OHSS undergo oocyte collection, cryopreserve of their embryos, and end the cycle, postponing embryo transfer until later.

These complications associated with the ART process (the need for numerous cycles to obtain a pregnancy, multiple-infant pregnancies, and the risks of OHSS) have been decreased by the growing use of cryopreservation in ART laboratories (Edgar et al., 2000; Oehninger et al., 2000; Schnorr et al., 2000; Edgar et al., 2005).

Cryopreservation is the preservation of cells, tissues, organs, or embryos by freezing. Since the early 1980's, ART has utilized this technology as a means to store embryos for future use (Trounson and Mohr, 1983). The implementation of cryopreservation has given ART clinics the freedom to use fewer embryos for transfer, as they are able to store the supernumerary embryos and use them later if pregnancy is not established (Edgar et al., 2005). Freezing provides an alternative for patients who may not wish to discard unused embryos, but who are also wary of a multiple-infant pregnancy and so do not wish to transfer all the embryos at once.

Even though cryopreservation is advantageous for patients, it has its own drawbacks. The 2005 National Summary of ART clinics in the United States reported that the percentage of transfers resulting in live births from fresh embryos in women under 35 years of age was about 43%, while only 32% of thawed embryos resulted in live births (Society for Assisted Reproductive Technology, 2006). However, some believe that the strict criteria used to choose fresh embryos for transfer gives an advantage to those embryos and, consequently, a disadvantage to those embryos left for cryopreservation (Edgar et al., 2000).

Edgar et al. (2000) found that nearly 45% of thawed embryos suffered some amount of blastomere (cell) loss, causing an approximate 30% reduction in implantation. They also suggested from these data that 80% of the resulting implantations arise from thawed embryos, which suffered no blastomere loss. Therefore, if methods can be altered in such a way as to protect the blastomeres from damage during cryopreservation/thawing, the patient should have an improved chance for implantation with the thawed embryos.

The purpose of this study was to determine if varying the temperatures at which cryopreserved embryos are thawed affects the rate of post-thaw blastocyst development.

Materials and Methods

Animals

The complete cryopreservation procedure for mouse embryos has been described earlier (Boone et al, 2004). Specific pathogen-free mice (B6C3F1) were obtained from Jackson Labs. Female mice were superovulated with intraperitoneal injection of 5.0 International Units (IU) of pregnant mare's serum gonadotropin (PMSG; Calbiochem, San Diego, CA). Each female was injected intraperitoneally with 5.0 IU human chorionic gonadotropin (hCG; Sigma Aldrich, St. Louis, MO) 48 hours after PMSG injection and immediately placed with a male. Females were sacrificed approximately 43 hours after hCG injection. Embryos were collected via oviductal lavage using approximately 0.1 mL of Dulbecco's phosphate-buffered saline. Only morphologically normal two-cell embryos were used in the study. All of these procedures complied with an approved Animal Research Committee protocol.

Cryopreservation

Embryos were cryopreserved in 1.5M propanediol and 0.1M Sucrose (SAGE In-Vitro Fertilization, Inc., Trumbull, CT), and subsequently frozen at the two-cell stage of development using the Sage Embryo Freeze protocol. Embryos were moved through three increasing levels of cryoprotectant to help remove water from the cells. Fifteen embryos were loaded into a plastic straw and heat sealed. A total of 16 straws were used in this experiment. Next, the straws were placed in a programmable freezer (Planer Freezer, Planer Co., UK) and cooled using the following settings: Ramp 1) 23°C to -6°C at minus 2°C per minute; Ramp 2) held at -6°C for 15 minutes for seeding (seeded 5 minutes into the ramp with 10 minutes post-seeding soak); Ramp 3) -6°C to -35°C at minus 0.3°C per minute; Ramp 4) held for 5 minutes; Ramp 5) -35°C to -180°C at minus 50°C per minute; and Ramp 6) held at -180°C for 5 minutes. The straws then were removed from the freezing chamber and plunged into liquid nitrogen for storage.

Thaw Procedure

Cryopreserved straws were thawed individually. Each was removed from the liquid nitrogen tank and allowed to thaw for 30 seconds at room temperature. The straw then was placed in a water bath for 30 seconds. Eight straws were thawed in water baths set at 30°C, and eight straws were thawed in water baths set at 37°C. Cryoprotectant was removed by exposing the embryos to decreasing concentrations of cryoprotectant (Quinn's Advantage Thaw Kit, SAGE In-Vitro Fertilization, Inc., Trumbull, CT). Upon removal of the cryoprotectants, the embryos were incubated for 72 hours and assessed for blastocyst development based on morphology.

Statistics

Proportions between blastocyst development for the embryos in the two different thaw temperatures were analyzed using the Chi square test.

Results

Blastocyst development for straws thawed at 30°C was 52.1% (63/121), compared with 45.5% (50/110) for straws thawed at 37°C. These two developmental rates were not statistically different ($P = 0.4$). Data are depicted in Table 1.

Figure 1 shows four embryos thawed in the course of this study. Embryo A is a blastocyst, characterized by the intact zona pellucida. Embryo B is an early blastocyst that has an extruded degenerated blastomere. Embryo C is a morula containing degenerated blastomeres. Embryo D is a degenerated four-cell embryo. This figure represents the type of embryos observed in this project. For our data, two of these embryos were classified as blastocysts and two as non-blastocysts.

Thaw Temperature (°C)	Non-blastocyst (%)	Blastocyst (%)	P value
30	47.9 (58/121)	52.1 (63/121)	0.4
37	54.5 (60/110)	45.5 (50/110)	

Table 1. Percent of post-thaw mouse blastocyst development after thawing in different temperatures.

Discussion

Cryopreservation has been a useful tool for infertile couples undergoing IVF-EC. However, thawed embryos have reduced rates of blastocyst development which adversely affects birth rates. The objective of this study was to assess the effect of different thaw temperatures on post-thaw blastocyst development.

Thaw procedures reported in other studies differ considerably. Valojerdi et al. (2002) reported thawing two-cell mouse embryos for 10 seconds at room temperature before shaking them in a 20°C water bath for 20 seconds and subsequently passing them through a series of dilution and rehydration steps. Another study reported thawing the

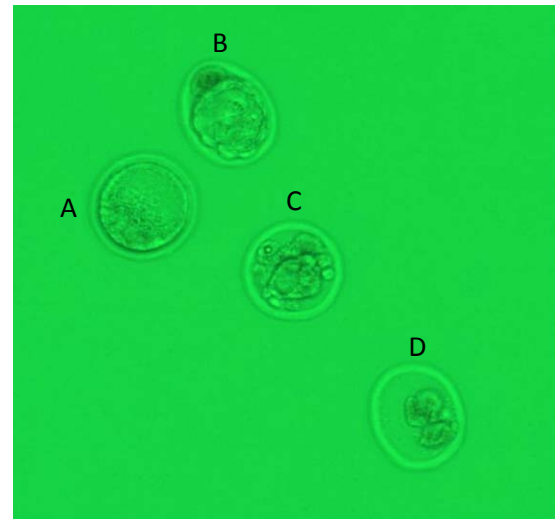


Figure 1. Development of four murine embryos 72 hours post-thaw.

vials in which the embryos were stored in a water bath at 31°C for 3 minutes before moving the embryos through a series of dilutions to remove the cryoprotectants (Burns et al., 1999). These two examples demonstrate how diverse the procedures are for thawing cryopreserved embryos, with a time difference of over two-and-one-half minutes and a temperature difference of 11°C.

For our study, murine embryos were cryopreserved and sequentially thawed in water baths at 30°C or 37°C for 30 seconds. Our results determined no significant difference in post-thaw murine blastocyst development between embryos thawed at 30°C and those thawed at 37°C. This may provide evidence to suggest that slightly varying thaw temperatures are inconsequential to the development of post-thaw blastocysts. However, many other aspects of the cryopreservation process should be assessed to determine their roles in affecting thawed-embryo development. For example, just as the time and temperature of reported thaw procedures varied, so do the dilution and rehydration steps. Perhaps if more accurate and precise methods can be developed for thawing cryopreserved embryos, subsequent thawed-embryo development rates, and ultimately birth rates, can be improved.

References

1. American Society for Reproductive Medicine (ASRM). 2004. Assisted Reproductive Technology in the United States: 2000 results generated from the American Society for Reproductive Medicine/Society for Assisted Reproductive Technology Registry. Fertility and Sterility. Vol. 81, No.5: 1207 – 1220.
2. Boone W.R., J.E. Johnson, D.W. Blackhurst, and H.L. Higdon III. 2004. Cryopreservation methodologies: freezing location and protocol alter development of mouse embryos. Contemporary Topics. Vol. 43: 26 – 31.
3. Burns, W.N., T.W. Gaudet, M.B. Martin, Y.R. Leal, H. Schoen, C.A. Eddy, and R.S. Schenken. 1999. Survival of cryopreservation and thawing with all blastomeres intact

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- identifies multicell embryos with superior frozen embryo transfer outcome. *Fertility and Sterility*. Vol. 72, No. 3: 527 – 532.
4. Centers for Disease Control and Prevention (CDC). 2006. 2004 Assisted Reproductive Technology Success Rates: National Summary and Fertility Clinic Reports.
 5. Chandra A, G.M. Martinez, M.D. Mosher, J.C. Abma, and J. Jones. 2005. Fertility, family planning, and reproductive health of U.S. women: Data from the 2002 National Survey of Family Growth. National Center for Health Statistics. *Vital Health Stat* Vol. 23, No.25.
 6. Chung, K., C. Coutifaris, R. Chalian, K. Lin, S. Ratcliffe, A.J. Castelbaum, M.F. Freedman, and K.T. Barnhart. 2006. Factors influencing adverse perinatal outcomes in pregnancies achieved through use of in vitro fertilization. *Fertility and Sterility*. Vol. 86, No. 6: 1634 – 1641.
 7. Cohen, J, and H.W. Jones Jr. 2001. How to avoid multiple pregnancies in assistive reproductive technologies. *Seminars in Reproductive Medicine*. Vol. 19, No. 3: 269 – 278.
 8. Edgar, D.H., H. Bourne, H. Jericho, and J.C. McBain. 2000. The developmental potential of cryopreserved human embryos. *Molecular and Cellular Endocrinology*. Vol. 169: 69 – 72.
 9. Edgar, D.H., J. Archer, and H. Bourne. 2005. The application and impact of cryopreservation of early cleavage stage embryos in assisted reproduction. *Human Fertility*. Vol. 8, No. 4: 225 – 230.
 10. Fasouliotis, S. J., and J.G. Schenker. 2002. Failures in assisted reproductive technology: an overview. *European Journal of Obstetrics & Gynecology and Reproductive Biology* Vol. 107: 4 – 18.
 11. Jones, H.W., H.J. Out, E.H. Hooman, S.G. Driessen, and H.J. Bennink. 1997. Cryopreservation: the practicalities of evaluation. *Human Reproduction*. Vol. 12, No. 7: 1522 – 1524.
 12. Lassalle, B., J. Testart, and J.P. Renard, 1985. Human embryo features that influence the success of cryopreservation with the use of 1,2-propanediol. *Fertility and Sterility*. Vol. 44, No. 5: 645 – 651.
 13. Nagy A, M. Gertsenstein, K. Vintersten, and R. Behringer. 2003. Manipulating the mouse embryo. A laboratory manual. Cold Spring Harbor Laboratory Press. Cold Spring Harbor, NY.
 14. Niemann, H. and B. Reichelt. 1993. Manipulating early pig embryos. *Journal of Reproduction and Fertility*. [Supplement] Vol. 48: 75 – 94.
 15. Oehninger, S., J. Mayer, and S. Muasher. 2000. Impact of different clinical variables on pregnancy outcome following embryo cryopreservation. *Molecular and Cellular Endocrinology*. Vol 169: 73 – 77.
 16. Queenan, J.T., L.L. Veeck, J.P. Toner, S Oehninger, and S.J. Muasher. 1997. Cryopreservation of all prezygotes in patients at risk of severe hyperstimulation does not eliminate the syndrome, but the chances of pregnancy are excellent with subsequent frozen-thaw transfers. *Human Reproduction*. Vol. 12, No. 7: 1573 – 1576.
 17. Schnorr, J.A., S.J. Muasher, and H.W. Jones Jr. 2000. Evaluation of the clinical efficacy of embryo cryopreservation. *Molecular and Cellular Endocrinology*. Vol. 169: 85 – 89.
 18. Senger, P.L. 2005. Pathways to Pregnancy and Parturition. 2nd Edition. Current Conceptions, Inc. Pullman, WA. 285 – 289, 348 – 365.
 19. Sher, G., V.M. Davis, and J. Stoess. 2005. In Vitro Fertilization: The A.R.T. of Making Babies. 3rd Edition. Checkmark Books. New York, NY. 63 – 65.
 20. Society for Assisted Reproductive Technology. 2006. IVF Success Rate Reports. www.sart.org.
 21. Trounson, A. and L. Mohr. 1983. Human pregnancy following cryopreservation, thawing and transfer of an eight-cell embryo. *Nature*. Vol. 305, No. 5936: 707 – 709.
 22. Valojerdi, M.R., M. Movahedin, and A. Hosseini. 2002. Improvement of Development of Vitirified Two-Cell Mouse Embryos by Vero Cell Culture. *Journal of Assisted Reproduction and Genetics*. Vol. 19, No. 1: 31 – 38.
 23. Wright, V.C, J. Chang, G. Jeng, and M. Macaluso. 2006. Assisted Reproductive Technology Surveillance – United States, 2003. National Center for Chronic Disease Prevention and Health Promotion (CDC). *Surveillance Summaries*. Vol. 55, No. SS04: 1-22.
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