Cryoloop vitrification in assisted reproduction: analysis of survival rates in >1000 human oocytes after ultra-rapid cooling with polymer augmented cryoprotectants

J. Liebermann[1], *Ph.D.*; **M. J. Tucker**[2, 3], *Ph.D.*; **E. S. Sills**[2, 3], *M.D.*

[1]*Department of Gynecology and Obstetrics, University of Würzburg, Würzburg (Germany)*
[2]*Georgia Reproductive Specialists LLC, Atlanta, Georgia*
[3]*Division of Reproductive Endocrinology and Infertility, Department of Obstetrics and Gynecology, Atlanta Medical Center, Atlanta, Georgia (USA)*

Summary

While human oocytes have been successfully cryopreserved using traditional slow-rate freezing protocols, inconsistent results post-thaw have limited the routine clinical application of oocyte cryopreservation. Despite interest in the potential benefits of vitrification as an alternative laboratory approach to long-term oocyte preservation in assisted reproduction, there is little agreement on how best to configure such cryopreservation protocols to optimize oocyte viability. To compare post-thaw oocyte survival rates, we performed cryoloop vitrification of human oocytes utilizing two different cryoprotectant mixtures that included polymer macromolecules. Human oocytes (n = 1120) were obtained from consenting patients undergoing in vitro fertilization, but only failed-matured (uninseminated) or failed-fertilized (inseminated but without 2*pn* development) were included in this investigation. Protocol A consisted of 20% ethylene glycol and 20% dimethyl sulphoxide + 0.4 M sucrose and 20% synthetic serum substitute. Protocol B consisted of 20% ethylene glycol and 20% dimethyl sulphoxide + 0.65 M sucrose, 1 mg/ml polyethylene glycol, 10 mg/ml Ficoll and 20% synthetic serum substitute. Following cryostorage for 10-14 d at –196°C, the survival rate for oocytes vitrified with protocol A was 80.9%, whereas the post-thaw viability among protocol B oocytes was 80.6% ($p > 0.005$). Our results indicate that an ethylene glycol + dimethyl sulphoxide mixture (with or without polymer macromolecules) can be an effective cryoprotectant strategy for human oocyte vitrification; either approach can be employed without any observed compromise in post-warming survival and/or morphology.

Key words: Human oocyte; Vitrification; Cryoloop; Polymer.

Introduction

A critical objective during mammalian oocyte and embryo cyopreservation is to reduce damage caused by intracellular ice formation. To achieve this goal, the two approaches of controlled slow-rate freezing [1] and rapid vitrification [2] have been developed. Prior investigators have shown that human oocytes can be cryopreserved using traditional slow-rate freezing protocols [3] with satisfactory obstetrical outcomes [4-8]. However, such deliveries account for a minority of in vitro fertilization-embryo transfer (IVF-ET) births. Inconsistent post-thaw outcomes have therefore sharply limited the application of oocyte cryopreservation as a routine laboratory technique.

To survive the stress associated with cryopreservation, the oocyte must tolerate a sequence of supraphysiologic volume changes (*i.e.*, contractions and expansions). Unlike all stages of the preimplantation human embryo, the oocyte is intrinsically susceptible to the effects of ice crystal formation associated with cryopreservation. This difference may be explained, at least in part, by the relatively low permeability of the ooplasmic membrane [9]. One way to avoid or minimize crystallization damage to human oocytes can be achieved by vitrification, which totally eliminates ice crystal formation-both within the oocyte itself (intracellular) and in the surrounding microenvironment (extracellular).

Vitrification (or "glass formation") refers to the solidification of a solution at a very low temperature, not by ice crystallization, but by extreme elevation in viscosity during cooling [10]. By using a small volume of high-concentration cryoprotectants, ultra-rapid cooling rates from 15,000 to 24,000°C/min can be achieved [11-13].

Thermodynamic management of the oocyte during vitrification involves technical challenges beyond those encountered in traditional cryologic procedures involving living cells. For example, osmotic changes associated with vitrification are often more severe than during conventional oocyte freezing, and the higher concentration of cryoprotectants required for vitrification can further amplify this toxic effect [14]. Removing the cryoprotectant (a necessary step to maintain normal cellular metabolic function) may also complicate osmoregulation during a period when the cell is particularly intolerant of such change. It is therefore not surprising that cryopreserved cells immediately post-thaw, in general, are more sensitive to osmotic swelling than fresh (non-cryopreserved) cells [15]. Although experience continues to develop in this area, some researchers have concluded that with sufficient technical skill, vitrified human

Revised manuscript accepted for publication February 18, 2003

oocytes can tolerate cryopreservation with less difficulty and are able to develop to blastocyst stage [16-19] and subsequently result in livebirths [20, 21].

The cryoloop [22-26] has been found to facilitate accelerated cooling in cryobiologic procedures. However, a direct comparison of different cryoloop-based vitrification protocols has not been reported for human oocytes. Furthermore, while inclusion of nonpermeable high molecular weight polymers into vitrification cryoprotectant regimens has been described, ours is the first formal assessment of these reagents specifically in an assisted reproduction context.

Materials and Methods

Human oocytes

The local ethics committee approved this investigation as well as the informed consent document signed by all study participants. Only oocytes demonstrating delayed maturation (uninseminated) and oocytes failing to fertilize normally (inseminated) after conventional IVF or intracytoplasmic sperm injection (ICSI) were included for analysis. Normal fertilization was confirmed by the presence of 2 pronuclei (*2pn*) by 30 h post-insemination, irrespective of technique used (conventional or ICSI).

Recognizing that oocytes at the germinal vesicle (GV), metaphase I (MI) or MII stages of maturity may have different potential for survival after vitrification, all oocytes were partitioned into those which were not injected (GV / MI; $n = 368$) resulting from preparation for ICSI only, and those that were inseminated by IVF or ICSI with at least one polar body (M II; $n = 752$). To reduce the influence of individual patient variation and assure that both experimental groups of oocytes were comparable in nature (differing only with respect to the vitrification protocol used), the failed-matured and failed-fertilized oocytes were segregated from normal fertilized oocytes. Next, all failed-matured and failed-fertilized oocytes were pooled and then randomly allocated to one of the two vitrification study protocols carried out in parallel. Warming of all oocytes was done concurrently using identical laboratory equipment.

To estimate the *a priori* background rate of oocyte degeneration independent of cryopreservation (*i.e.*, loss due to intrinsic attributes of failed-fertilized oocytes), we reviewed laboratory data from IVF experience at this institution for the six-month period ending December 2001. These data formed an oocyte control group ($n = 810$), from which we calculated an overall oocyte degeneracy rate of 1.6% at 24 h post-insemination (13/810). Additionally, fractured and empty zonae were found to occur in about 0.6% of oocytes (6/810); the rate of degenerate oocytes was 0.9% (7/810).

Vitrification and dilution solutions

Chemicals for pre-equilibration, vitrification and dilution (Sigma; St. Louis, MO, USA) were prepared with Dulbecco's phosphate-buffered saline (DPBS) without calcium or magnesium (IVC, In-Vitro Care; San Diego, CA, USA) plus 20% serum substitute supplement (SSS, Irvine Scientific; Santa Ana, CA, USA) and IVC-1 culture medium. For this investigation, all oocytes were vitrified using a 20 mm cryoloop with diameter of ~0.5 mm (Hampton Research; Laguna Niguel, CA, USA).

Our vitrification procedure commenced in culture dishes (stock #150326, Nalge Nunc International; Copenhagen, Denmark) prepared with 100 µl holding medium (HM)(DPBS + 20% SSS), 100 µl vitrification solution 10 (VS10)(10% (v/v) ethylene glycol [EG] + 10% (v/v) dimethyl sulphoxide [DMSO]) and 100 µl vitrification solution 20 (VS20)(20% (v/v) EG + 20% (v/v) DMSO) with an additional 20 µl-droplet of vitrification soulution 20. For the warming procedure, a 6-well tissue culture plate (Genesis Instruments; Elmwood, WI, USA) was prepared with 1 ml of each dilution solution. Post-warming solutions were comprised of DPBS + 20% SSS at different concentrations of sucrose.

Cryoloop vitrification technique

For all oocytes, cryoloop vitrification was performed according to the method described by Lane *et al.* [22, 23]. In brief, oocytes were placed in HM x 10 min at room temperature, and then placed in VS10, and finally transferred into VS20. During this time the nylon loop was dipped into VS20 to create a thin, filmy layer of the VS20 on the nylon loop surface. After a short exposure to VS20, oocytes were next placed onto the nylon loop pre-loaded with the thin film of VS20 using a fine pulled-glass capillary tube. The loaded nylon loop was then plunged vertically into liquid nitrogen (-196°C). Standard aluminum cryologic canes were used for cell storage under liquid nitrogen.

Experimental design

In pre-trials, the influence of the loading temperature (25°C vs 37°C) vis-à-vis oocyte survival was evaluated. When incubated in the vitrification solution for 30-60 sec at 37°C, no oocyte demonstrated normal re-expansion post-thaw. Based on this finding, it was determined that a loading temperature near room temperature (25°C) would be used uniformly for this investigation. A conspectus of each vitrification solution is presented in Table 1.

Oocytes were assigned to one of the following cryoloop vitrification protocols. Protocol A: After 10 min of pre-equilibration in HM, oocytes were incubated with 10% EG + 10% DMSO x ~60sec. While oocytes were incubated in VS10, the cryoloop was placed into VS20 to create a thin, filmy layer of the solution on the nylon loop. Next, oocytes were transferred to 20% EG, 20% DMSO + 0.4M sucrose x ~30sec at 25°C (*i.e.*, exposure time of oocytes to VS20 = 30sec). Protocol B: After 10 min of pre-equilibration in HM, oocytes were first exposed to VS10 with polymers (1 mg/ml PEG; mol.wt. = 8,000D) x ~60sec, followed by transfer to VS20 with polymers (1 mg/ml polyethylene glycol [PEG] + 10 mg/ml Ficoll 400 [Pharmacia

Table 1. — *Summary of laboratory protocols utilized for human oocyte vitrification by protocol A (n = 429) and protocol B (n = 691).*

Protocol	Pre-equilibration	Vitrification solution 10	Vitrification solution 20	Warming solution
A	20%SSS x10min	10% EG, 10% DMSO x60sec	20% EG, 20% DMSO, 0.4M S x30sec	1M S, 0.5M S, 0.25M S, 0.125M S
B	20%SSS x10min	10% EG, 10% DMSO 1mg/ml PEG x60sec	20% EG, 20% DMSO, 1mg/ml PEG, 10 mg/ml F x30sec	1M S, 0.5M S, 0.25M S, 0.125M S

EG = ethylene glycol; DMSO = dimethyl sulphoxide; F = Ficoll 400; PEG = polyethylene glycol; S = sucrose; SSS = serum substitute supplement.

AB; Stockholm, Sweden]; mol.wt. = 400,000D) plus 0.65M sucrose x 30 sec at 25°C (*i.e.*, exposure time of oocytes to VS20 + polymers = 30 sec).

After cryostorage for 10-14 d, all vitrified oocytes were simultaneously warmed and diluted using a uniform four-step dilution with sucrose on a 37°C warm heat stage. Briefly, the cryoloops were removed from liquid nitrogen and with the cryovial still submerged, the vial was opened and the nylon loop containing oocytes was extracted. Subsequently, the nylon loop was placed above the warming solution (1M sucrose diluted in DPBS + 20% SSS). As the nylon loop contents liquefied, the loop was promptly submerged in the 1M sucrose droplet where oocytes immediately fell from the loop. After 2 min, oocytes were transferred to 0.5 M sucrose x 3 min, and sequentially to 0.25 M and finally 0.125 M sucrose, being exposed for 5 min in each solution, and finally washed x 2 in HM.

Post-thaw assessment of human oocytes and statistical analysis

After warming, the recovered oocytes were placed in 20 µl IVC-1 under mineral oil at 37°C in a humidified atmosphere of 5% CO2 in air x 24 h. Oocytes were then evaluated under a stereomicroscope. For this investigation, oocytes that were morphologically competent (*i.e.*, intact zonae pellucidae and plasma membrane with a refractive homogeneous cytoplasm, and relatively clear perivitelline space) after the dilution of the cryoprotectant followed by 24-h culture were considered viable. In contrast, ocytes with fragmented cytoplasm, indistinct oolemma, damaged zona pellucida, and/or oocytes that degenerated during the 24-h culture period were recorded as nonviable. Statistical analysis (χ^2 test) was carried out by means of computer-assisted data software (Microsoft Excel 2001; Redmond, WA, USA). Differences between groups were considered significant when $p < 0.05$.

Results

A comparision of oocyte survival rates after the two experimental vitrification protocols employed in this investigation is shown in Table 2. Failure of fertilization was noted in 752 human oocytes, and of these 304 were processed without polymer macromolecule supplementation. The remaining 448 oocytes underwent cryopreservation with macromolecule-augmented cryoprotectant. The observed survival rate for these failed-fertilized oocytes after 24 h of culture was similar, irrespective of the macromolecule component in the cryoprotectant solution (80.9 vs 80.6%; $p > 0.005$). Weighted-average calculation of these data demonstrated an overall survival rate among failed-fertilized oocytes of 80.7%.

When we observed failed-matured human oocytes ($n = 368$) undergoing vitrification according to the two experimental protocols, again no significant difference in viability was found after 24 h of culture (71.2 vs 73.3%; $p > 0.005$). Weighted-average calculation of this data subset demonstrated an overall survival rate among failed-matured oocytes of 72.6%. From this, a significantly lower post-thaw survival rate among failed-matured oocytes as compared to failed-fertilized oocytes was evident (72.6 vs 80.7%; $p = 0.025$).

Table 2. — *Post-thaw survival of vitrified human oocytes (*n = *1120) after 24 h* in vitro *culture as a function of developmental stage and cryologic method.*

	Failed-fertilized oocytes		Failed-matured oocytes	
Vitrification protocol	A	B	A	B
n	304	448	125	243
Oocyte survival (%)	246 (80.9)[a]	361 (80.6)[a]	89 (71.2)[b]	178 (73.3)[b]
Overall survival (%)	607/752 (80.7)[c]		267/368 (72.6)[c]	

[a]$p > 0.05$; [b]$p > 0.05$; [c]$p = 0.025$; all comparisons by χ^2 test.

Discussion

The aim of this investigation was to explore the efficacy of a mixture of EG and DMSO, with or without high molecular weight polymers in the vitrification solution. Additionally, we sought to characterize the impact of developmental stage of the human oocyte (*i.e.*, inseminated but failed-fertilized and uninseminated secondary to failed-maturation) on survival following vitrification. In this study, our results describe a post-thaw survival of selected human oocytes of approximately 80% with or without non-permeating solutes such as Ficoll 400 and PEG. Furthermore, we found oocytes were tolerant of brief exposure (30 sec) to 20% EG + 20% DMSO mixture at room temperature without any apparent deleterious effect. That oocyte viability was preserved despite brief exposure to EG and DMSO was unexpected, since the permeability of the cytoplasmic membrane of the oocyte is substantially lower than that of the human embryo [9].

While other investigators have reported satisfactory survival rates (up to 65%) following vitrification of oocytes when high cryoprotectant concentrations were used [16-20], ours is the first study to examine survival rates with these two vitrification protocols in more than 1,000 human oocytes. Indeed, very high post-warming survival rates of 91% [27] and even 96% [17] have been reported in studies of more limited oocyte populations ($n < 65$). More recently, an investigation of human oocyte cryopreservation utilizing a slow-rate protocol achieved a similar survival rate when the oocytes were frozen with 0.2 mol/l sucrose, and the survival rate was even higher when the sucrose concentration was 0.3 mol/l (60 vs 82%) [3].

What is the physiologic basis for using macromolecule-augmented cryoprotectants? Cells naturally contain higher concentrations of protein than the extracellular space. Thus, a relatively higher concentration of cryoprotectants is needed to achieve extracellular as compared to intracellular vitrification. A negative consequence of this is that with some cryoprotectants, this minimal concentration (C_v) can lead either to osmotic or chemical toxicity. The addition of polymers of high molecular weight such as polyvinylpyrrolidone, PEG or Ficoll 400 may be beneficial in promoting extracellular vitrification while maintaining constant cryoprotectant concentrations intracellularly. Previous work has suggested that addition of polymers to the cryoprotectant milieu may reduce the C_v by as much as 24%, when combined with an increased hydrostatic pressure [10].

Accordingly, the vitrification solution used in protocol B was supplemented with PEG and Ficoll 400, both high

molecular weight polymers. Prior studies using embryos (rather than oocytes) have assessed the impact of adding such macromolecular solutes to the vitrification solution [28-32]. Such polymers are thought to protect embryos against cryoinjury by mitigating stress occurring during cooling [29] accomplished by viscosity modification; this is thought to reduce significantly the amount of cryoprotectant required to achieve vitrification [31]. Furthermore, polymers may form a protective viscous matrix encapsulating the cell, further preventing ice crystallization during cooling and warming [28, 32].

O'Neill *et al.* [30] observed that addition of PEG resulted in improved viability of murine oocytes following vitrification and reduced the variability seen when using vitrification solutions without macromolecular augmentation. In contrast, Shaw *et al.* [31] concluded that Ficoll 400 had only a negligible effect on the glass transition of cryoprotectants. Another more recent study evaluated cryoprotectant solutions consisting of high polymer concentrations with low concentrations of penetrating cryoprotectant, finding high rates of development of 2-cell mouse embryos after rapid cooling and warming [32].

To increase oocyte membrane permeability, we incubated oocytes for ~10min in DPBS with 20% SSS, a biologic macromolecule. The rationale behind this pre-equilibration was to permit adequate penetration of the permeating mixture of EG/DMSO. With respect to this pre-equilibration time (and its potential adverse impact on oocyte spindle structure), in parallel studies we vitrified discarded oocytes but without pre-equilibration. Observations from these preliminary studies are similar to data presented here, suggesting that the 10 min pre-equilibration phase is not necessarily required for post-warming survival (data not shown). Protocol B consisted of a slightly higher sucrose concentration (0.65M) compared with protocol A (0.4M). Yet, the fact that survival rates with both procedures were similar indicates that this slight difference did not impact post-thaw outcome. Likewise, our results suggest that the addition of macromolecular polymers (PEG or Ficoll 400) to the vitrification solution has little effect on post-thaw oocyte survival.

To date, there has been little agreement on whether failed-matured human oocytes would demonstrate any difference in survival after vitrification, when compared to failed-fertilized oocytes. Given that aqueous membrane permeability determines the dehydration behavior of oocytes during the vitrifying process, it is plausible that both uninseminated and inseminated (but unfertilized) oocytes would behave similarly in this regard. Since oocytes were stratified according to developmental stage in our investigation, the study design permitted detailed analysis of this matter. In this experiment we found the post-warming survival among immature oocytes to be significantly less ($p = 0.025$) than the survival rate achieved with failed-fertilized human oocytes.

The 80.7% survival for failed-fertilized oocytes and 72.6% survival for failed-matured oocytes registered in our laboratory may be considered a satisfactory survival rate for both groups of human oocytes following cryopreservation. However, these findings should be interpreted with caution since our investigation was confined only to those oocytes that failed to mature or fertilize normally. It cannot be known if such a high level of post-thaw viability would necessarily apply to oocytes with normal maturation and fertilization characteristics. Additionally, it must be acknowledged that these oocytes did not prove their competence by maturing or fertilizing *in vitro*. Nevertheless, given the necessary ethical constraints on research involving human gametes we believe that the oocytes studied here do provide insights for refinement of vitrification approaches in human-assisted reproduction. Whether normal, non-discarded oocytes would yield acceptable cryosurvival with application of these protocols remains open for study, and forms the basis of ongoing research at our institutions.

References

[1] Whittingham D.G., Leibo S.P., Mazur P.: "Survival of mouse embryos frozen to -196 degrees and -269 degrees C". *Science*, 1972, *178*, 411.

[2] Rall W.F., Fahy G.M.: "Ice-free cryopreservation of mouse embryos at –196 °C by vitrification". *Nature*, 1985, *313*, 573.

[3] Fabbri R., Porcu E., Marsella T., Rocchetta G., Venturoli S., Flamigni C.: "Human oocyte cryopreservation: new perspectives regarding oocyte survival". *Hum. Reprod.*, 2001, *16*, 411.

[4] Chen C.: "Pregnancy after human oocyte cryopreservation". *Lancet*, 1986, *1*, 884.

[5] Van Uem J.F.H.M., Siebzehnrubl E.R., Schuh B., Koch R., Trotnow S., Lang N.: "Birth after cryopreservation of unfertilized oocytes". *Lancet*, 1987, *1*, 752.

[6] Porcu E., Fabbri R., Seracchioli R., Ciotti P.M., Magrini O., Flamigni C.: "Birth of a healthy female after intracytoplasmic sperm injection of cryopreserved human oocytes". *Fertil. Steril.*, 1997, *68*, 724.

[7] Tucker M.J., Morton P.C., Wright G., Sweitzer C.L., Massey J.B.: "Clinical application of human egg cryopreservation". *Hum. Reprod.*, 1998, *13*, 3156.

[8] Tucker M.J., Wright G., Morton P.C., Massey J.B.: "Birth after cryopreservation of immature oocytes with subsequent in vitro maturation". *Fertil. Steril.*, 1998, *70*, 578.

[9] Ruffing N.A., Steponkus P.L., Pitt R.E., Parks J.E.: "Osmometric behavior, hydraulic conductivity, and incidence of intracellular ice formation in bovine oocytes at different developmental stages". *Cryobiology*, 1993, *30*, 562.

[10] Fahy G.M., McFarlane D.R., Angell C.A., Meryman H.T.: "Vitrification as an approach to cryopreservation". *Cryobiology*, 1984, *21*, 407.

[11] Martino A., Songsasen N., Leibo S.P.: "Development into blastocysts of bovine oocytes cryopreserved by ultra-rapid cooling". *Biol. Reprod.*, 1996, *54*, 1059.

[12] Arav A., Zeron Y.: "Vitrification of bovine oocytes using modified minimum drop size technique (MDS) is effected by the composition and concentration of the vitrification solution and by the cooling conditions". *Theriogenology*, 1997, *47*, 341.

[13] Vajta G., Booth P.J., Holm P., Greve T., Callesen H.: "Successful vitrification of early stage bovine in vitro produced embryos with the Open Pulled Straw (OPS) method". *CryoLetters*, 1997, *18*, 191.

[14] Quinn P., Kerin J.F.: "Experience with the cryopreservation of human embryos using the mouse as a model to establish successful techniques". *J. In Vitro Fert. Embryo Transf.*, 1986, *3*, 40.

[15] Pedro P.B., Zhu S.E., Makino N., Sakurai T., Edashige K., Kasai M.: "Effects of hypotonic stress on the survival of mouse oocytes and embryos at various stages". *Cryobiology*, 1997, *35*, 150.

[16] Hunter J.E., Fuller B.J., Bernard A., Jackson A., Shaw R.W.: "Vitrification of human oocytes following minimal exposure to cryoprotectants; initial studies on fertilization and embryonic development". *Hum. Reprod.*, 1995, *10*, 1184.

[17] Chen S.U., Lien Y.R., Chao K.H., Lu H.F., Ho H.N., Yang Y.S.: "Cryopreservation of mature human oocytes by vitrification with ethylene glycol in straws". *Fertil. Steril.,* 2000, *74,* 804.
[18] Chung H.M., Hong S.W., Lim J.M., Lee S.H., Cha W.T., Ko J.J.: "In vitro blastocyst formation of human oocytes obtained from unstimulated and stimulated cycles after vitrification at various maturational stages". *Fertil. Steril.,* 2000, *73,* 545.
[19] Wu J., Zhang L., Wang X.: "In vitro maturation, fertilization and embryo development after ultrarapid freezing of immature human oocytes". *Reproduction,* 2001, *121,* 389.
[20] Kuleshova L., Gianaroli L., Magli C., Ferraretti A., Trounson A.: "Birth following vitrification of a small number of human oocytes: case report". *Hum. Reprod.,* 1999, *14,* 3077.
[21] Yoon T.K., Chung H.M., Lim J.M., Han S.Y., Ko J.J., Cha K.Y.: "Pregnancy and delivery of healthy infants developed from vitrified oocytes in a stimulated in vitro fertilization-embryo transfer program". *Fertil. Steril.,* 2000, *74,* 180.
[22] Lane M., Schoolcraft W.B., Gardner D.K.: "Vitrification of mouse and human blastocysts using a novel cryoloop container-less technique". *Fertil. Steril.,* 1999, *72,* 1073.
[23] Lane M., Bavister B.D., Lyons E.A., Forest K.T.: "Container-less vitrification of mammalian oocytes and embryos". *Nat. Biotechnol.,* 1999, *17,* 1234.
[24] Mukaida T., Nakamura S., Tomiyama T., Wada S., Kasai M., Takahashi K.: "Successful birth after transfer of vitrified human blastocysts with use of a cryoloop containerless technique". *Fertil. Steril.,* 2001, *76,* 618.
[25] Oberstein N., O'Donovan M.K., Bruemmer J.E., Seidel G.E. Jr., Carnevale E.M., Squires E.L.: "Cryopreservation of equine embryos by open pulled straws, cryoloop, or conventional cooling methods". *Theriogenology,* 2001, *15,* 607.
[26] Yeoman R.P., Gerami-Naini B., Mitalipov S., Nusser K.D., Widmann-Browning A.A., Wolf D.P.: "Cryoloop vitrification yields superior survival of Rhesus monkey blastocysts". *Hum. Reprod.,* 2001, *16,* 1965.
[27] Kuwayama M., Kato O.: "Successful vitrification of human oocytes". *Fertil. Steril.,* 2000, *74* (Suppl. 3), S49.
[28] Kasai M., Komi J.H., Takakamo A., Tsudera H., Sakurai T., Machida T.: "A simple method for mouse embryo cryopreservation in a low toxicity vitrification solution, without appreciable loss of viability". *J. Reprod. Fertil.,* 1990, *89,* 91.
[29] Dumoulin J.C., Bergers-Janssen J.M., Pieters M.H., Enginsu M.E., Geraedts J.P., Evers J.L.: "The protective effects of polymers in the cryopreservation of human and mouse zonae pellucidae and embryos". *Fertil. Steril.,* 1994, *62,* 793.
[30] O'Neill L., Paynter S.J., Fuller B.J.: "Vitrification of mature mouse oocytes: improved results following addition of polyethylene glycol to a dimethyl sulfoxide solution". *Cryobiology,* 1997, *34,* 295.
[31] Shaw J.M., Kuleshova L.L., MacFarlane D.R., Trounson A.O.: "Vitrification properties of solutions of ethylene glycol in saline containing PVP, Ficoll, or dextran". *Cryobiology,* 1997, *35,* 219.
[32] Kuleshova L.L., Shaw J.M., Trounson A.O.: "Studies on replacing most of the penetrating cryoprotectant by polymers for embryo cryopreservation". *Cryobiology,* 2001, *43,* 21.

Address reprint requests to:
E. S. SILLS, M.D., FACOG, FACS
Suite 270
5445 Meridian Mark Road
Atlanta, Georgia 30342 (USA)