Human Reproduction Vol.20, No.2 pp. 492–496, 2005 doi:10.1093/humrep/deh605
Advance Access publication November 3, 2004.

Aseptic technology of vitrification of human pronuclear oocytes using open-pulled straws

V.Isachenko[1,3], M.Montag[1], E.Isachenko[1], V.Zaeva[2], I.Krivokharchenko[2], R.Shafei[2] and H.van der Ven[1]

[1]Department of Gynaecological Endocrinology and Reproductive Medicine, University of Bonn, Bonn, Germany and [2]Gynaecological Clinic 'Ma-Ma', Moscow, Russia

[3]To whom correspondence should be addressed at: Department of Gynaecological Endocrinology and Reproductive Medicine, University of Bonn, Sigmund-Freud-Str. 25, D-53105 Bonn, Germany. E-mail: vovaisachenko@yahoo.com

BACKGROUND: The aim of this study was to compare the viability of human pronuclear oocytes subjected to vitrification using cooling by direct submerging of open-pulled straws in liquid nitrogen versus vitrification by cooling of open-pulled straws located inside a closed 0.5 ml straw (aseptic system). METHODS: Two- and three-pronuclei stage oocytes ($n = 114$) were cryopreserved in super-open-pulled straws by vitrification in 20% ethylene glycol + 20% dimethylsulphoxide (DMSO) + osmotic active and neutral non-permeable cryoprotectants with a four-step exposure in 20, 33, 50 and 100% vitrification solution for 2, 1 and 1 min, and 30–50 s, respectively at room temperature, and plunging into liquid nitrogen. Oocytes of group 1 ($n = 42$) were rapidly cooled at a speed of 20 000°C/min by direct plunging of open-pulled straws into liquid nitrogen. Oocytes of group 2 ($n = 44$) were first located in 0.5 ml straws, which were closed at both sides by metal balls, and then plunged into liquid nitrogen. This method resulted in a cooling speed of 200°C/min. For both groups, oocytes were thawed rapidly at a speed of 20 000°C/min using an identical protocol. Oocytes subsequently were expelled into a graded series of sucrose solutions (1.0, 0.75, 0.5, 0.25 and 0.12 mol/l) at 2.5 min intervals. RESULTS: Oocyte development up to expanded blastocyst stage after *in vitro* culture was 15% in group 1, 14% in group 2 and 29% in an untreated control group. CONCLUSION: The deposition of human pronuclear oocytes in open-pulled straws which are placed inside a hermetically closed container guarantees a complete isolation of oocytes from liquid nitrogen and avoids potential contamination by pathogenic microorganisms. The combination of direct plunging of this container into liquid nitrogen and rapid warming makes this process as efficient as conventional vitrification.

Key words: aseptic application/microbial contamination/oocytes/open-pulled straw/vitrification

Introduction

There have been several recent reports of the successful cryopreservation of human pronuclear oocytes by direct plunging into liquid nitrogen (vitrification) (Park *et al.*, 2000; Jelinkova *et al.*, 2002; Liebermann and Tucker, 2002; Liebermann *et al.*, 2002a,b; Selman and El-Danasouri, 2002; Isachenko *et al.*, 2004a).

For the cryopreservation of embryos or oocytes, standard 0.25 ml insemination straws were used almost exclusively (Kuleshova and Shaw, 2000). The achievable cooling rate by direct plunging into liquid nitrogen (Palasz and Mapletoft, 1996) was ~ 2500°C/min. However, new methods based on the immersion of very small amounts of solutions in liquid nitrogen were developed and most authors have attributed the improved developmental rate of cells after cryopreservation to an increased (>10 000°C/min) speed of freezing and warming (Martino *et al.*, 1996; Vajta *et al.*, 1998; Lane *et al.*, 1999; Lane and Gardner, 2001; Dinnyes *et al.*, 2000; Kuwayama and Kato, 2000).

All methods mentioned above have a common parameter: exposure of the vitrification solution holding the biological object directly to liquid nitrogen during freezing. Isolation of vitrifying samples from liquid nitrogen in order to avoid direct contact results in a decreased speed of cooling which, in the opinion of the majority of authors, can decrease the efficacy of the vitrification protocol.

Bielanski *et al.*, (2000) reported the contamination of bovine embryos by viral pathogens during storage of these embryos in liquid nitrogen. Even the vapour of liquid nitrogen can be contaminated by microorganisms (Fountain *et al.*, 1997). Consequently, long-term banking of spermatozoa and embryos in liquid nitrogen can have a potential risk of cross-contamination when specimens are not sealed properly (Bielanski *et al.*, 2003).

The aim of our investigations was to assess the viability of vitrified human pronuclear oocytes after slow cooling of samples which were protected from direct contact with liquid nitrogen followed by rapid warming.

Materials and methods

Except where otherwise stated, all chemicals were obtained from Sigma (Sigma Chemical Co., St Louis, MO). Written informed consent was obtained from the participating couples for the cryopreservation and culture of oocytes. The study was performed in Russia and obtained approval from the State Ethics Committee.

Patients with unexplained infertility were stimulated for IVF-ICSI with triptorelin (Decapeptyl®; Ferring, Kiel, Germany) and recombinant FSH (Puregon®; Organon, Oss, The Netherlands) according to the 'long' protocol. Ovulation was induced by the administration of 10 000 IU of HCG (Pregnil®; Organon). Oocytes were retrieved 34–36 h later and inseminated with the husband's sperm through conventional IVF or ICSI techniques. Fertilization was assessed 14–18 h after insemination. Abnormally fertilized oocytes [at the three-pronuclei (PN) stage, $n = 102$] or 2PN stage (from patients who did not want cryopreservation of oocytes, $n = 12$) were used for vitrification, warming and subsequent culture.

In total, 62 patients ranging in age from 24 to 39 years (median age 32 ± 3.9) volunteered to have their oocytes cryopreserved by vitrification at the 2PN or 3PN stage.

Twelve oocytes with 2PN and 102 oocytes with 3PN were assigned randomly to a control ($n = 28$) and two experimental groups. As we found no difference in the developmental rate of embryos derived from these two stages, all data were combined and presented together. The oocytes in the experimental groups were non-equilibrium cryopreserved (vitrified) by plunging into liquid nitrogen. All the cryopreservation solutions were prepared in TCM-199 supplemented with 20% serum supplement (Irvine Scientific, Santa Aria, CA). Oocyte cryopreservation was performed in open-pulled-straws (OPS) (Medical Technology GmbH, Altdorf, Germany) (Vajta *et al.*, 1998) with a four-step exposure in 20, 33, 50 and 100% vitrification solution [20% ethylene glycol +20% dimethylsulphoxide (DMSO) +0.75 mol/l sucrose) for 2, 1 and 1 min, and 30–50 s, respectively at 37°C and cooled. The volume of final vitrification solution in the OPS was 2–2.5 µl. For group 1, rapid cooling of oocytes ($n = 42$) was realized by direct plunging of OPS into liquid nitrogen. For group 2, OPS with oocytes ($n = 44$) were first placed inside a sterile 0.5 ml insemination straw (Medical Technology GmbH), which was hermetically closed by metal balls at both sides (Figure 1), and then plunged into liquid nitrogen. It should be noted that there was no contact between the metal ball and the vitrification solution holding the oocytes due to the presence of a meniscus of the vitrification solution (Figure 1). The potential risk of contact of vitrification solution with the metal ball is not a big problem because the metal balls and 0.5 ml straws are sterile; oocytes locate in the upper part of a column of vitrification solution (Figure 1). The speed of cooling in 'straw in straw' vitrification was determined using a Testo 950 electrical thermometer (Testo AG, Lenzkirch, Germany). For rapid warming, the OPS of group 1 were removed from liquid nitrogen and immediately plunged into a 1.5 ml microcentrifuge tube (Eppendorf-Netheler-Hinz GmbH, Hamburg, Germany) containing 1 ml of 1 mol/l sucrose. Before plunging into sucrose solution, the OPS of group 2 were first removed from the 0.5 ml straws in the following way. On removal of the ball using scissors, the part of the 0.5 ml straw which was still half submerged in liquid nitrogen is exposed and then, using fine forceps, OPS were taken out of the 0.5 ml straw and plunged into

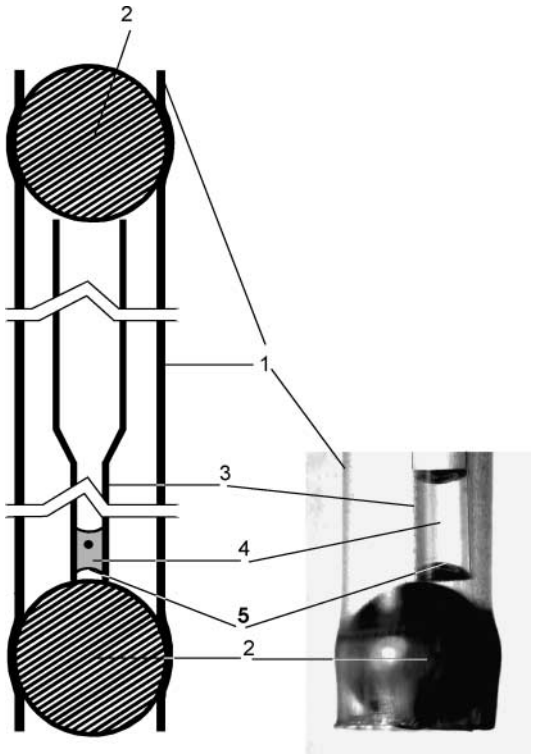

Figure 1. Scheme and photographs of the container for vitrification using the 'straw in straw' mode: (1) 0.5 ml straw; (2) metal closing ball; (3) open-pulled straw; (4) vitrification medium with oocyte; (5) meniscus of vitrification medium.

sucrose solution as described above. This mode of thawing allows for the simultaneous removal of cryoprotectant and rapid warming. After warming, oocytes of both groups were expelled into a graded series of sucrose solutions (1.0, 0.75, 0.5, 0.25 and 0.12 mol/l) for gradual removal of the cryoprotectant in five 2.5 min steps. All

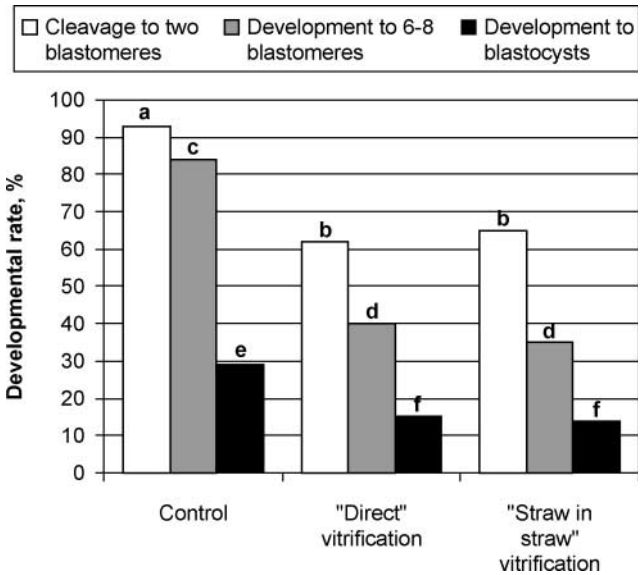

Figure 2. Development of oocytes after vitrification with direct cooling of open-pulled straws (OPS) in liquid nitrogen versus vitrification using cooling of OPS previously located in hermetically closed 0.5 ml straws. Superscripts a–b, c–d and e–f indicate significant differences ($P < 0.05$).

the oocytes were then cultured for an additional 6 days in 5% CO_2 at 37°C in Blast-Assist Medium (I and II) (Medicult, Denmark) according to a standard protocol.

Results were analysed by logistic regression with dummy variables using the LOGISTIC procedure of SAS (SAS Stat Software, 1996). Statistical significance was denoted as $P < 0.05$.

Results

In the control group, 25 out of 28 (93%) fresh oocytes were viable and cleaved to the two-blastomere stage (Figure 2). The developmental rate to the 6–8 blastomere stage and to blastocysts was 84 and 29%, respectively.

For experimental group 1 (vitrification with cooling by direct plunging into liquid nitrogen), the rates of formation of the 2-cell embryo, 6–8 blastomere embryo and blastocysts were 62, 40 and 15%, respectively. For group 2 (vitrification with cooling of OPS located in a 0.5 ml straw), these rates were 65, 35 and 14%, respectively (Figure 2), and not significantly different from group 1.

Figure 3 shows the appearance of the vitrified pronuclear oocytes subjected to vitrification using OPS located inside the closed 0.5 ml straw. Just after warming, a dispersion of nucleoli was occasionally detected; later nucleoli were re-organized to a more polarized pattern and the initial dispersion had no influence on the further development of embryos.

Discussion

There are two different technologies for cooling of cells prior to storage in liquid nitrogen: cooling by direct plunging into liquid nitrogen (at a temperature of >1000–10 000°C/min; Isachenko *et al.*, 1997, 2000, 2001, 2003a,b, 2004a; Vajta *et al.*, 1998) and cooling in vapour of liquid nitrogen (at ~200°C/min; Szell *et al.*, 1990; Rall, 1993; Darvelid *et al.*, 1994; Rall and Wood, 1994; Dinnyes *et al.*, 1995; Vajta *et al.*, 1996; Mukaida *et al.*, 1998; Donnay *et al.*, 1998; Yokota *et al.*, 2001). This second methodology of 'slow'

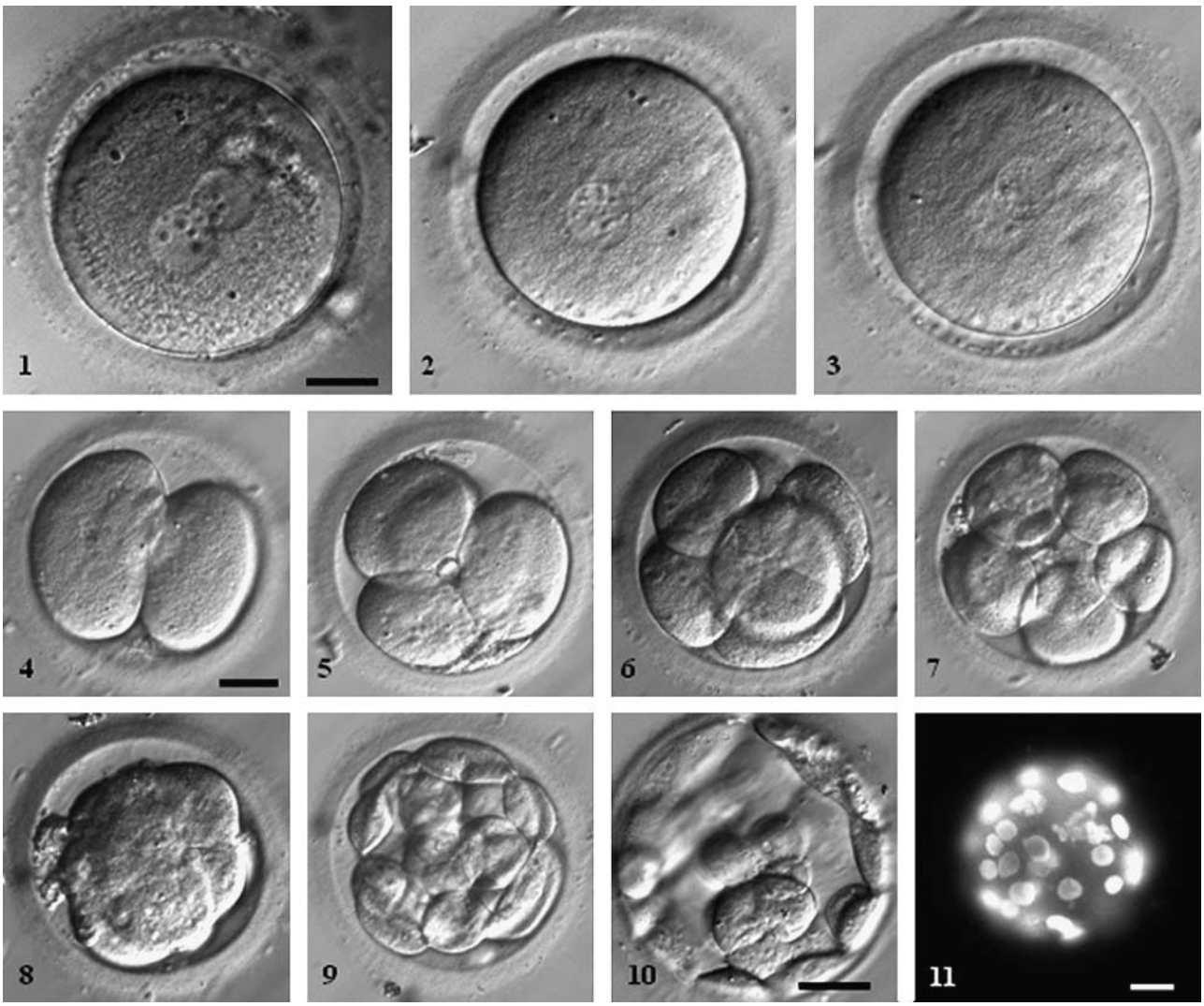

Figure 3. The same 2PN oocyte before vitrification (1), and after warming: for 5 min (2 and 3 at a different focus), 6 h (4), 18 h (5), 30 h (6), 42 h (7), 66 h (8), 78 h (9), 96 h (10) and 96 h, stained by Hoechst 33342 (11). Bar = 30 μm.

cooling of cells prior to storage in liquid nitrogen is another proof of the secondary role of this parameter for the vitrification process.

All combinations of 'slow' or 'quick' cooling with 'slow' warming were the main cause for a decreasing viability of vitrified mouse embryos (Rall and Fahy, 1985; Rall, 1987; Rall and Wood, 1994).

Elevated warming rates and the absence of visible crystal formation during thawing of oocytes was shown to be most effective when the warming/cooling proportion was >1.3. For example, cooling at 200°C/min followed by warming at 360°C/min (warming/cooling proportion 1.3) is a more effective regime than cooling at 2000°C/min followed by warming at 2400°C/min (warming/cooling proportion 1.2) (Isachenko *et al.*, 1998, 1999).

In the opinion of the majority of authors, the high effectiveness of different protocols for the vitrification of oocytes and embryos with decreased volumes of cooled medium can be explained by the combination of high speeds of cooling and warming (Martino *et al.*, 1996; Vajta *et al.*, 1998; Lane *et al.*, 1999; Lane and Gardner, 2001; Mukaida *et al*., 2003; Son *et al.*, 2003; Vanderzwalmen *et al.*, 2003). The results of our investigations with human oocytes do not support this point of view. For human oocytes, the relatively slow cooling rate in combination with rapid warming is as efficient as 'conventional' vitrification with regard to survival rates and embryo development.

Cremades *et al.*, (2004) reported the successful vitrification of human embryos using fine diameter plastic micropipettes, which were cooled in the vapour of liquid nitrogen and subsequently placed inside a pre-cooled cryotube. Similarly to our findings, this methodology also includes the parameter 'slow cooling' and 'rapid warming'; however, this methodology does not prevent the contact with nitrogen of an open system holding the biological objects.

'Slow' cooling of biological objects can also be used for cooling of a relatively large volume. To prevent the 0.25 ml straw from coming into direct contact with liquid nitrogen and eliminating the potential contamination risk associated with storage in liquid nitrogen, Kuleshova and Shaw (2000) have reported 'straw in straw' vitrification of mouse embryos. In their protocol, a standard 0.25 ml straw containing vitrification medium was located inside another 0.5 ml straw which was hermetically closed before plunging into liquid nitrogen. However, this method, in contrast to the one described herein, does not allow for rapid warming of a small volume and simultaneous removal of the cryoprotectant during the thawing process. Other data rgarding 'straw in straw' cryopreservation of ovine germinal vesicle stage oocytes (Isachenko *et al.*, 2001) and mouse embryos (Walker *et al.*, 2004) have also been published.

Our finding regarding the minor importance of the speed of cooling for the vitrification of oocytes and embryos can probably be applied to all reproductive human cells including spermatozoa. We recently reported that vitrification of human spermatozoa by fast (20 000°C/min) or relatively slow (200°C/min) cooling resulted in similar post-thaw characteristics (Isachenko *et al.*, 2004b).

The major point of the work presented here is that the described technique completely avoids direct contact between liquid nitrogen and the oocytes. In fact, any technology in reproductive biology and especially in a therapeutic medical approach must ensure and guarantee the full protection of biological objects from microorganisms (Gardner, 1998; Bielanski *et al.*, 2000). Liquid nitrogen, which is used for storage of frozen material, can be a source of contamination by these microorganisms (Tedder *et al.*, 1995; Bielanski *et al.*, 2000). Filtration or ultraviolet treatment of liquid nitrogen cannot guarantee the absence of contamination of biological material by viruses including human immunodeficiency virus (HIV). For example, Tedder *et al.* reported the contamination of blood probes by hepatitis virus during the time of storage of probes in liquid nitrogen (Tedder *et al.*, 1995). Different types of viruses, which are simple and very cryo-stable structures, may increase their virulence after direct plunging and storage in liquid nitrogen, like hepatitis virus (Hawkins *et al.*, 1996), papova virus (Charles and Sire, 1971), vesicular stomatitis virus (Schaffer *et al.*, 1976) and herpes virus (Jones and Darville, 1989).

In conclusion, vitrification of human pronuclear oocytes in OPS which are placed inside a hermetically closed container before plunging into liquid nitrogen allows a reliable isolation of oocytes from liquid nitrogen and avoids contamination by pathogenic microorganisms. Although this technique is associated with a relatively slow cooling rate, the developmental potential of these pronuclear oocytes is not compromised if the thawing process involves rapid warming and simultaneous removal of cryoprotectants.

Acknowledgements

The authors wish to thank Dr S.Zaletov (Clinic 'Ma-Ma', Moscow, Russia) for help with the organization of the experiments.

References

Bielanski A, Nadin-Davis S, Sapp T and Lutze-Wallace C (2000) Viral contamination of embryos cryopreserved in liquid nitrogen. Cryobiology 40,110–116.

Bielanski A, Bergeron H, Lau PCK and Devenish J (2003) Microbial contamination of embryos and semen during long term banking in liquid nitrogen. Cryobiology 46,146–152.

Charles GN and Sire DJ (1971) Transmission of papova virus by cryotherapy applicator. J Am Med Assoc 218,1435.

Cremades N, Sousa M, Silva J, Viana P, Sousa S, Olivera C, Teixeira da Silva J and Barros A (2004) Experimental vitrification of human compacted morulae and early blastocysts using fine diameter plastic micropipettes. Hum Reprod 19,300–305.

Darvelid U, Gustafsson H, Shamsuddin M, Larsson B and Rodriquez-Martinez H (1994) Survival rate and ultrastructure of vitrified bovine in vitro and in vivo developed embryos. Acta Vet Scand 35,417–426.

Dinnyes A, Wallace GA and Rall WF (1995) Effect of genotype on the efficiency of mouse embryo cryopreservation by vitrification or slow freezing. Mol Reprod Dev 40,429–435.

Dinnyes A, Dai Y, Jiang S and Yang X (2000) High development rates of vitrified bovine oocytes following parthenogenetic activation, in vitro fertilization, and somatic cell nuclear transfer. Biol Reprod 63,513–518.

Donnay I, Auquier P, Kaidi S, Carolan C, Lonergan P, Mermillod P and Massip A (1998) Vitrification of in vitro produced bovine blastocysts: methodological studies and developmental capacity. Anim Reprod Sci 52, 93–104.

Fountain D, Ralston M, Higgins N, Gorlin JB, Uhl L, Wheeler C, Antin JH, Churchill WH and Benjamin RJ (1997) Liquid nitrogen freezers: a potential

sourse of microbial contamination of hematopoietic stem cell components. Transfusion 37,587–591.

Gardner DK (1998) Development of serum-free media for the culture and transfer of human blastocysts. Hum Reprod 13,218–285.

Hawkins AE, Zuckerman MA, Briggs M, Gilson RJ, Goldstone AH, Brink NS and Tedder RS (1996) Hepatitis B nucleotide sequence analysis: linking an outbreak of acute hepatitis B to contamination of a cryopreservation tank. J Virol Methods 60,81–88.

Isachenko V, Isachenko E, Ostashko F and Grishchenko V (1997) Ultrarapid freezing of rat embryos with rapid dilution of permeable cryoprotectants. Cryobiology 34,157–164.

Isachenko V, Perez-Sanchez F, Isachenko V, Grishchenko V and Soler C (1998) Vitrification of GV-porcine oocytes with intact intracellular lipids: effect of the cryoprotectant saturation/dilution stepping, elevated temperature and cytoskeletal inhibitor. Cryobiology 36,250–253.

Isachenko V, Gorbunov L, Isachenko E, Ostashko F and Bezugly N (1999) Some physical and technological aspects of GV porcine oocyte vitrification. Cryobiology 39, 35 abstract.

Isachenko V, Alabart JL, Isachenko E, Michelmann HW and Bezugly N (2000) Ultra-rapid freezing and storage of rat embryos in an electric refrigerator at −130°C without liquid cryo-agents with ultra-short exposure in the freezing medium and direct rehydration after thawing. CryoLetters 21,13–18.

Isachenko V, Alabart JL, Nawroth F, Isachenko E, Vajta G and Folch J (2001) The open pulled straw vitrification of ovine GV-oocytes: positive effect of rapid cooling or rapid thawing or both? CryoLetters 22,157–162.

Isachenko V, Folch J, Nawroth F, Krivokharchenko A, Vajta G, Dattena M and Alabart JL (2003a) Double vitrification of rat embryos at different developmental stages using an identification protocol. Theriogenology 60, 445–452.

Isachenko V, Selman H, Isachenko E, Montag M, El-Danasouri I and Nawroth F (2003b) Modified vitrification of human pronuclear oocytes: efficacy and effect on ultrastructure. RBM Online 7,211–216.

Isachenko V, Montag M, Isachenko E, Nawroth, Dessole S and Van der Ven H (2004a) Developmental rate and ultrastructure of vitrified human pronuclear oocytes after step-wise versus direct rehydration. Hum Reprod 19,660–665.

Isachenko V, Isachenko E, Katkov II, Montag M, Dessole S, Nawroth F and Van der Ven H (2004b) Cryoprotectant-free cryopreservation of human spermatozoa by vitrification and freezing in vapor: effect on motility, DNA integrity, and fertilization ability. Biol Reprod 71,1167–1173.

Jelinkova L, Selman HA, Arav A, Strehler E, Reeka N and Sterzik K (2002) Twin pregnancy after vitrification of 2-pronuclei human embryos. Fertil Steril 77,412–414.

Jones SK and Darville JM (1989) Transmission of virus-particles by cryotherapy and multi-use caustic pencils: a problem to a dermatologist? Br J Dermatol 121,481–486.

Kuleshova LL and Shaw JM (2000) A strategy for rapid cooling of mouse embryos within a double straw to eliminate the risk of contamination during storage in liquid nitrogen. Hum Reprod 15,2604–2609.

Kuwayama M and Kato O (2000) Successful vitrification of human oocytes. Fertil Steril 74,549.

Lane M, Bavister BD, Lyons EA and Forest KT (1999) Containerless vitrification of mammalian oocytes. Nat Biotechnol 17,1234–1236.

Lane M and Gardner DK (2001) Vitrification of mouse oocytes using a nylon loop. Mol Reprod Dev 58,342–347.

Liebermann J and Tucker MJ (2002) Effect of carrier system on the yield of human oocytes and embryos as assessed by survival and developmental potential after vitrification. Reproduction 124,483–489.

Liebermann J, Tucker MJ, Graham JR, Han T, Davis A and Levy MJ (2002a) Blastocyst development after vitrification of multipronuclear zygotes using the Flexipet denuding pipette. RBM Online 4,148–152.

Liebermann J, Nawroth F, Isachenko V, Isachenko E, Rahimi G and Tucker M (2002b) Potential importance of vitrification in reproductive medicine. Biol Reprod 67,1671–1680.

Martino A, Songansen N and Leibo SP (1996) Development into blastocysts of bovine oocytes cryopreserved by ultra-rapid cooling. Biol Reprod 54, 1059–1069.

Mukaida T, Wada M, Takahashi K, Pedro PB, An TZ and Kasai M (1998) Vitrification of human embryos based on the assessment of suitable conditions for 8-cell mouse embryos. Hum Reprod 13,2874–2879.

Mukaida T, Nakamura S, Tomiyama T, Wada S, Oka C, Kasai M and Takahashi K (2003) Vitrification of human blastocysts using cryoloops: clinical outcome of 223 cycles. Hum Reprod 18,384–391.

Palasz AT and Mapletoft RJ (1996) Cryopreservation of mammalian embryos and oocytes: recent advances. Biotechnol Adv 14,127–149.

Park SP, Kim EY, Oh JH, Nam HK, Lee KS, Park SY, Park EM, Yoon SH, Chung KS and Lim JH (2000) Ultra-rapid freezing of human multipronuclear zygotes using electron microscope grids. Hum Reprod 15,1787–1790.

Rall WF (1987) Factors affecting the survival of mouse embryos cryopreserved by vitrification. Cryobiology 24,387–402.

Rall WF (1993) Advances in the cryopreservation of embryos and prospects for alication to the conservation of salmonid fishes. In Cloud JG and Thorgaard GH (eds), Genetic conservation of salmonid fishes. Plenum Press, New York, pp. 137–158.

Rall WF and Fahy GM (1985) Ice-free cryopreservation of mouse embryos at −196°C by vitrification. Nature 313,573–575.

Rall WF and Wood MJ (1994) High in vitro and in vivo survival of day 3 mouse embryos vitrified in a non-toxic solution of glycerol and albumin. J Reprod Fertil 101,681–688.

SAS Stat Software (1996) Changes and enhancement through release 6.11. SAS Institute, Cary, NC.

Schaffer TW, Everen J, Silver GH and Came PE (1976) Biohazard: virus-contaminated liquid nitrogen. Science 192,25–26.

Selman HA and El-Danasouri I (2002) Pregnancies derived from vitrified human zygotes. Fertil Steril 77,422–423.

Son WY, Yoon SH, Yoon HJ, Lee SM and Lim JH (2003) Pregnancy outcome following transfer of human blastocysts vitrified on electron microscopy grids after induced collapse of the blastocoele. Hum Reprod 18,137–139.

Szell A, Zhang J and Hudson R (1990) Rapid cryopreservation of sheep embryos by direct transfer into liquid nitrogen vapour at − 180°C. Reprod Fertil Dev 2,613–618.

Tedder RS, Zuckerman MA, Goldstone AH, Hawkins AE, Fielding A, Briggs EM, Irwin D, Blair S, Gorman AM and Patterson KG et al. (1995) Hepatitis-B transmission from contaminated cryopreservation tank. Lancet 346, 137–140.

Vajta G, Holm P, Greve T and Callesen H (1996) Factors affecting survival rates of in vitro produced bovine embryos after vitrification and direct in-straw rehydration. Anim Reprod Sci 45,191–200.

Vajta G, Kuwayama M, Holm P, Booth PJ, Jacobsen H, Greve T and Callesen H (1998) Open pulled straw (OPS) vitrification: a new way to reduce cryoinjuries of bovine ova and embryos. Mol Reprod Dev 51,53–58.

Vanderzwalmen P, Bertin G, Debauche Ch, Standaert V, Bollen N, Rosendaal van E, Vandervorst M, Schoysman R and Zech N (2003) Vitrification of human blastocysts with hemi-straw carrier: application of assisted hatching after thawing. Hum Reprod 18,1504–1511.

Walker D, Tummon S, Hammitt DG, Session DR, Dumesic P and Thornhill A (2004) Vitrification versus programmable rate freezing of late stage murine embryos: a randomized comparison prior to application in clinical IVF. RBM Online 8,558–568.

Yokota Y, Sato S, Yokota M and Araki Y (2001) Birth of a healthy baby following vitrification of human blastocysts. Fertil Steril 75,1027–1029.

Submitted on July 12, 2004; resubmitted on August 13, 2004; accepted on October 15, 2004