—Mini Review—

Evidence-based Embryo Cryopreservation

Masashige Kuwayama[1]

[1]*Kato Ladies Clinic, Advanced Medical Research Institute of Fertility, 7-20-3, Nishishinjyuku, Shinjyuku, Tokyo 160-0023, Japan*

Abstract: *In 1972, cryopreservation of mammalian animal embryos was developed using mouse embryos. To utilize surplus embryos for in vitro fertilization programs, this technique was applied to human embryos by the slow freezing method in the first half of the 1980's, and by vitrification in the 1990's. Recently, the protocol of vitrification of human embryos has been improved by the ultra-rapid vitrification method in which the rate of cooling is highly improved. It is mainly used as an effective basic technique for cryopreservation of human embryos in current clinical IVF programs due to the extremely high embryonic survival rate after storage.*

Key words: *Cryopreservation, Slow freezing, Vitrification, Human embryo, IVF*

Cryopreservation of mammalian animal embryos facilitates the storage of the genetic resources of specific animals or species. This technique is essential for the improvement of breed and husbandry of domestic animals, and is required for maintaining zoo and wild animal species that are in danger of becoming extinct. In the medical field, as a basic technique for infertility treatment programs, cryopreservation of embryos has commonly been employed to utilize surplus embryos for in vitro fertilization (IVF)s. In this study, we review the development of cryopreservation of mammalian animal embryos, it's application in human IVF programs, and recent aspects of vitrification.

Text

Smith *et al*. [1, 2] initially stored rabbit embryos successfully in glycerol, which had been reported to protect sperm in freezing [3], for implantation studies. Twenty years later, Whittingham *et al.* [4] and Wilmut [5] employed dimethyl sulfoxide (DMSO), with a molecular structure resembling that of glycerol, as a cryoprotectant, and reported that mouse embryos frozen and stored by slow freezing showed a high survival rate after fusion. Based on their successful results, studies regarding cryopreservation of mammalian animal embryos rapidly accelerated, and this procedure was applied to the cryopreservation of the ova and embryos of small laboratory animals (rats [6, 7], rabbits [8–10]).

For domestic animals, Wilmut & Rowson [11] initially reported the application of procedure in cows, then sheep [12–14] and goats [15]. Since the publication of these reports, the research and development of cryopreservation of embryos, as a basic technique for embryo implantation, have been extensively performed for storing and transporting bovine embryos with a good hereditary disposition. The advances in embryo implantation-associated techniques were driven by global commercial factors [16–28].

Based on these studies, Trounson, who managed the Animal Reproduction and Human Reproduction Sections in Monash University, based on bovine cryopreservation methods, developed an optimal protocol for slow freezing of human embryos, and a frozen embryo-derived neonate was born, which was the first case of IVF treatment after cryopreservation in glycerol [29]. Thereafter, this procedure was standardized using propandiol, which was reported as effective in bovine embryos was reported by Suzuki [30], and sucrose, which has marked osmotic pressure-buffering effects as reported by Leibo [23, 24], and it has been employed in infertility hospitals throughout the world with the widespread use of a programmable freezer.

Vitrification was theoretically proposed by Luyet [31]. An intracellular/extracellular solution is solidified with no ice crystals like glass by rapid cooling, and living cells are in liquid nitrogen. As embryos are rapidly cooled without an expensive cooling system, this procedure was considered an ideal technique; however, actual

Received: January 18, 2005
Accepted: February 5, 2005
e-mail: masaabc@bekkoame.ne.jp

successful results were not obtained for a long while [32–35]. Procedures using a high concentration of cryoprotective agents facilitated vitrification of vegetable cells [36] and insect cells [37]. Subsequently, Rall and Fahy [38] reported the vitrification of mouse 8-cell stage embryos was successful, leading to the current vitrification techniques of embryos which are characterized by a high survival rate, simplicity and short time procedure.

After the success of Rall and Fahy, many studies investigated the vitrification technique in embryos from various animal species, Sheffen *et al.* [39] reported the successful vitrification of mouse embryos using a vitrification solution with a simpler composition. In addition, vitrification was successful in mice [40, 41], rats [42, 43], and rabbits [44–47], among small laboratory animals, as well as in sheep [48] and pigs [49–51], among domestic animals. In cows, Massip *et al.* [52] applied the method described by Sheffen *et al.* [39] in mice, and they were the first to achieve survival after vitrification using in vivo morula. Also, Kuwayama [53] was successful using IVF-derived blastocysts. Furthermore, Kuwayama [54] first showed electron microscopy that germinal intracellular/extracellular solutions were completely vitrified by in the freeze replica method.

Initial vitrification was developed using embryos derived from different animal species or embryos at different developmental stages in the 1990's, using vitrification solutions in which 50% v/v or more of cryoprotective agents were added, and a toxic vitrification solution with an osmotic pressure of 8,000 mosmol or more was used. Furthermore, this protocol, involving insufficient equilibrium and dilution, was applied to human embryos without optimization, and it's effectiveness was compared with the results of slow freezing. However, survival rates exceeding those obtained using the conventional method, that is, a survival rate of more than 90% after preservation, could not be obtained [55–60].

Vajta *et al.* [61, 62], developed the open pulled straw (OPS) method, which employs rapid vitrification using a plastic straw finely processed after heating the end. Kuleshova *et al.* [63] achieved neonates derived from human oocytes that had been vitrified using this method. However, as was demonstrated for the gel loading tip method using a commercially available tip [64], and for the microdroplet method [65] in which microdroplets are dropped into liquid nitrogen, these rapid cooling methods did not achieve a sufficient rate of cooling to obtain a high survival rate; at issue was the cellular survival after vitrification.

In 1996, Martino *et al.* [66] prevented chilling injury in the ice temperature range by the ultra-rapid cooling vitrification procedure, in which a bovine ovum after equilibrium of vitrification solution was placed on the grid of an electron microscope, and directly added to liquid nitrogen for cooling, and they reported a high survival rate after preservation. Since then, a procedure in which the rate at which specimens are cooled is accelerated by minimizing the volume of the vitrification solution to be cooled, has been mainly employed, and a high survival rate is obtained by promoting intracellular vitrification. Improvement in the rate of cooling may promote vitrification, decreasing the vitrification fluid level of a cryoprotectant, and inhibiting toxicity to the minimum. The nylon loop method reported by Lane and Gardner [67] achieves vitrification of a micro volume of vitrification solution and embryos in a small nylon thread ring. A Cryoloop [68] (proprietary name) was used for vitrification of human blastocysts and achieved post-thaw survival rates corresponding to those for the conventional slow freezing. Similarly, using bovine embryos, the minimum volume cooling (MVC) straw method [69], in which an embryo is applied to the external straw wall, or the internal wall of a cut-opened straw for ultra-rapid vitrification, after equilibrium with vitrification solution was developed. This method decreases the concentration of membrane permeable cryoprotective agents to 30%, and the osmotic pressure to 4,000 mosmol, which corresponds to 50% of the values found in conventional vitrification solutions. The minimization of the only negative factor in the protocol of vitrification, solution toxicity, achieved a markedly high post-thaw survival rate for human embryos. The ultra-rapid vitrification procedure has been clinically applied using a special vitrification container (CryoTop, Kitazato Supply Co., Ltd.), which was developed for the clinical application of the MVC method [70]. This procedure is beginnings to be commonly utilized as extremely effective cryopreservation techniques for human embryos which maintains embryonic viability after storage and thawing.

Conclusion

The CryoTop method, a vitrification method in which the protocol was optimized is the most effective cryopreservation method for human embryos, and has been employed in approximately 230 IVF hospitals in 8 countries over the past 5 years. Regardless of the pronucleus, cleavage, and blastocyst stages, a high

survival rate of approximately 100% after vitrification is achieved, and cryo-blastocyst 1-embryo transfer, in which transfer is performed in the cycle with a better uterine condition, not in the cycle of ovum collection, achieves a high rate of pregnancy (42%). As a way of solving the serious issue of multiple pregnancies induced by conventional ART, the necessity of this procedure has recently been emphasized. In the future, in a large number of additional studies, this procedure should be investigated as a cryopreservation method essential for cryo-blastocyst 1-embryo transfer, without significant reduction of embryonic activity.

References

1) Smith, A.U. (1952): Behavior of fertilized rabbit eggs exposed to glycerol and to low temperatures. Nature, 170, 374–375.
2) Smith, A.U. (1953): In vitro experiments with rabbit eggs. Mammalian Ger Found Symp., pp. 217–222, London.
3) Polge, C., Smith, A.U. and Parkes, A.S. (1949): Revival of spermatozoa after vitrification and dehydration at low temperature. Nature, 164, 666.
4) Whittingham, D.G., Leibo, S.P. and Mazure, P. (1972): Survival of mouse embryos frozen to –196 and –269°C. Science, 178, 411–414.
5) Wilmut, I. (1972): The effect of cooling rate, warming rate, cryoprotective agent and stage of developement on survival of mouse embryos during freezing and thawing. Life Sci., 11, 1071–1079.
6) Utsumi, K. and Yuhara, M. (1974): Survival of rat embryos after freesing and thawing. Jpn. J. Infertility, 20, 20.
7) Whittengham, D.G. (1975): Survival of rat embryos after freezing and thawing. J. Reprod. Fert., 43, 575–578.
8) Bank, H. and Maurer, R.R. (1974): Survival of frozen rabbit embryos. Exp. Cell Res., 89, 188–196.
9) Whittingham, D.G. and Adams, C.E. (1976): Low temperature preservation of rabbit embryos. J. Reprod. Fert., 47, 269–274.
10) Tsunoda, Y. and Sugie, T. (1977): Survival of rabbit eggs preserved in plastic straws in liquid nitrogen. J. Reprod. Fert., 49, 173–174.
11) Wilmut, I. and Rowson, L.E.M. (1973): Experiments on the low temperature preservation of cow eggs. Vet. Rec., 92, 686–690.
12) Willadsen, S.M., Polge, C., Rowson, L.E.A. and Moor, R.M. (1974): Preservation of sheep embryos in liquid nitrogen. Cryobiology, 11, 560 (Abstr.).
13) Willadsen, S.M. (1976): Deep Freezing of sheep embryos. J. Reprod. Fert., 46, 151–154.
14) Schwier, M.C., Rall, W.F., Stuart, L.D. and Wildt, D.E. (1990): In situ straw dilution of ovine embryos cryopreserved by conventional freezing or vitrification. Theriogenology, 33, 321 (Abstr.).
15) Bilton, R.J. and Moore, N.W. (1976): In vitro culture, storage and transfer of goat embryos. Aust. J. Biol. Sci., 29, 125–129.
16) Bilton, R.J. and Moore, N.W. (1976): Storage of cattle embryos. J. Reprod. Fert., 46, 537.
17) Trounson, A.O., Shea, B.F., Ollis, G.W. and Jacobson, M.A. (1978): Frozen storage and transfer of bovine embryos. J. Anim. Sci., 47, 677–681.
18) Willadsen, S.M., Polge, C. and Rowson, L.E.A. (1978): The viability of deep-frozen cow embryos. J. Reprod. Fert., 52, 391–393.
19) Wiladsen, S.M. (1980): Deep freezing of embryos in the large domestic species. 9th Int. Congre. Anim. Reprod. & Artif. Insemi., RT-F-4, 255–261.
20) Leibo, S.P. and Mazur, P. (1978): Mathod of preservation of mammalian embryos by freezing. In: Methods in Mammalian Reproduction (Daniel, J.C., ed) pp. 179–201, Academic Press, New York.
21) Niemann, H., Sacher, B., Schilbing, E. and Smidt, D. (1982): Improvement of survival rates of bovine blastocysts with sucrose for glycerol dilution after a fast freezing and thawing method. Theriogenology, 17, 102 (Abstr.).
22) Renard, J.P., Heyman, Y. and Ozil, J.P. (1982): Congelation de l'embryon bovin: usenouvelle methode de decongelation pour le transfert cervical d'embryons conditionnes une seule fois en paillettes. Ann. Med. Vet., 126, 25–32.
23) Leibo, S.P. (1983): A one-step method for direct nonsurgical transfer of frozen thawed embryos. Theriogenology, 54, 427–432.
24) Leibo, S.P. (1983): A one-step in situ dilution method for frozen-thawed bovine embryos. Cryo-Lett., 4, 387–400.
25) Leibo, S.P. (1984): A one-step method for direct nonsurgical transfer of frozen thawed embryos. Theriogenology, 21, 767–789.
26) Renard, J.P. and Heyman, Y. (1983): Effet de mode et de dilution du cryoprotecteur sur la viabilite des blastocystes de vache apres decongelation. 16th Internat. Congr. Refrigerratiuon Commission, C-266, 25–32.
27) Massip, A. and Van. Der. Zwalmen, P. (1984): Direct transfer of frozen cow embryos in glycerol-sucrose. Vet. Rec., 115, 327–328.
28) Chupin, D., Florin, B. and Procureur, R. (1984): Comparison of two methods for one-step in-straw thawing and direct transfer of cattle blastocysts. Theriogenology, 21, 455–459.
29) Trounson, A. and Mohr, L. (1983): Human pregnancy following cryopreservation, thawing and transfer of an eight-cell embryo. Nature, 305, 707–709.
30) Suzuki, T., Yamamoto, M., Ooe, M., Sakata, A., Matsuoka, M., Nishikata, Y. and Okamoto, K. (1990): Effect of sucrose concentrations used for one-step dilution upon in vivo survival of bovine embryos refrigerated in glycerol and 1, 2-propanediol. Theriogenology, 34, 1051–1057.
31) Luyet, B.J. (1937): The vitrification of organic colloids and of protoplasm. Biodynamica, 29, 1–14.
32) Luyet, B.J. (1966): Anatomy of the freezing process in physical systems. In: Cryobiology (Meryman, H.T., ed.),

pp. 115–138, Academic Press, London.
33) Rapatz, G.L., Menz, L.J. and Luyet, B.J. (1966): Anatomy of the freezing process in physical systems. In: Cryobiology (Meryman, H.T., ed.), pp. 139–162, Academic Press, London.
34) Fahy, G.M., MacFarlane, D.R., Angell, C.A. and Meryman, H.T. (1984): Vitrification as an approach to cryopreservation. Cryobiology, 21, 407–426.
35) Fahy, G.M., McGrath, J.J. and Diller, K.R. (1988): Low Temperature Biotechnology Emerging Applications and Engineering Contributions. Amer. Soc. Mech. Eng. BED., 1, 113–146.
36) Grout, B.W. and Henshaw, G.G. (1978): Freeze preservation of potato shoot-tip cultures. Ann. Bot., 42, 1227–1229.
37) James, E. (1980): Cryopreservation of Schistosoma mansoni schistosomula using 40% v/v methanol and rapid cooling. Cryo-Letters, 1, 535–544.
38) Rall, W.F. and Fahy, G.M. (1985): Ice-free cryopreservation of mouse embryos by vitrification. Nature, 313, 573–575.
39) Scheffen, B., Van. Der. Zwalmen., P. and Massip, A. (1986): A simple and efficient procedure for preservation of mouse embryos by vitrification. Cryo-Letters, 7, 260–269.
40) Hsu, T.T., Yamakawa, H., Yamanoi, J. and Ogawa, S. (1986): Survival and transfer test of mouse early embryos frozen by vitrification. Jpn. J. Anim. Reprod., 32, 29–32.
41) Kasai, M., Komi, J.H., Takakamo, A. Tsudera, H., Salurai, T. and Machida, T. (1990): A simple method for mouse embryo cryopreservation in a low toxicity vitrification solution, without appreciable loss of viavility. J. Reprod. Fert., 89, 91–97.
42) Ischenko, V.V., Ostashko, F.I. and Isachenko, E.F. (1992): Vitrification and ultra-rapid freezing of rat embryos. Theriogenology, 37, 227.
43) Nakamichi, R., Ohboshi, S. and Fujihara, N. (1993): Vitrification of rat blastocysts developed in vitro. Zygote, 1, 281–285.
44) Kobayashi, K., Nagashima, H., Yamakawa, H., Kato, Y. and Ogawa, S. (1990): The survival of whole and bisected rabbit morulae after cryopreservation by vitrification method. Theriogenology, 33, 777–788.
45) Smorag, Z. and Gadja, B. (1991): Vitrification of non-cultured and cultured rabbit embryos. Anim. Reprod. Sci., 26, 151–158.
46) Kasai, M., Hamaguchi, Y., Zhu, S.E., Miyake, T., Sakurai, T. and Machida, T. (1992): High survival of rabbit morulae after vitrification in an ethylene glycol-based solution by a simple method. Biol. Reprod., 46, 1042–1046.
47) Papis, K., Fujikawa, S., Kojima, T. and Oguri, N. (1993): Effect of the composition of vitrification media on survival of rabbit embryos. Cryobiology, 30, 98–105.
48) Martinez, A.G. and Matkovic, M. (1998): Cryopreservation of ovine embryos: slow freezing and vitrification. Theriogenology, 1, 1039–1049.
49) Yoshino, J., Kojima, T., Shimizu, M. and Tomizuka, T. (1993): Cryopreservation of porcine blastocysts by vitrification. Cryobiology, 30, 413–422.
50) Kobayashi, S., Takei, M., Tomita, M. and Leibo, SP. (1998): Piglets produced by transfer of vitrified porcine embryos after stepwise dilution of cryoprotectants. Cryobiology, 36, 20–31.
51) Dobrinsky, J.R. and Johnson, L.A. (1994): Cryopresrvation of porcine embryos by vitrification: a study of in vitro development. Theriogenology, 42, 25–35.
52) Massip, A., Van. Der. Zwalmen, P., Scheffen, B. and Ectors, F. (1986): Pregnancies following transfer of cattle embryos preserved by vitrification. Cryo-Letters, 7, 270–273.
53) Kuwayama, M., Hamano, S. and Nagai, T. (1992): Vitrification of bovine blastocysts obtained by in vitro culture of oocytes matured and fertilized in vitro. J. Reprod. Fert., 96, 187–193.
54) Kuwayama, M., Fujikawa, S. and Nagai, T. (1994): Ultrastructure of IVM-IVF bovine blastocysts vitrified after equilibration in glycerol 1, 2- propanediol using 2-step and 16-step procedures. Cryobiology, 31, 415–422.
55) Gordts, S., Roziers, P. and Campo, R. (1990): Survival and pregnancy outcome after ultrarapid freezing of human embryos. Fertil. Steril., 53, 469–472.
56) Barg, P.E., Barad, D.H. and Feichtinger, W. (1990): Ultrarapid freezing of mouse and human preembryos: A modified approach. J. In Vitro Fert. Embryo Transfer, 6, 355–357.
57) Feichtinger, W., Hochfellner, C. and Ferstl, U. (1991): Clinical experience with ultrarapid freesing of embryos. Hum. Reprod., 6, 735–736.
58) Hsieh, Y.Y., Tsai, H.D. and Cjang, C.C. (1999): Ultrarapid cryopreservation of human embryos: experience with 1,582 embryos. Fertil. Steril., 72, 253–256.
59) Mukaida, T., Wada, S. and Takahashi, K. (1998): Vitrification of human embryos based on the assessment of suitable conditions for 8-cell mouse embryos. Hum. Reprod., 13, 2874–2879.
60) Yokota, Y., Sato, S. and Yokota, M. (2000): Successful pregnancy following blastocyst vitrification. Hum. Reprod., 15, 1802–1803.
61) Vajta, G., Kuwayama, M., Holm, P., Booth, PJ., Jacobson, H., Greve, T. and Callesen, H. (1998): Open plled straw vitrification:a new way to reduce cryoinjuries of bovine ova and embryos. Mol. Reprod. Dev., 51, 33–58.
62) Vajta, G., Lewis, I.M., Kuwayama, M. Greve, T. and Callesen, H. (1998): Sterile application of the open pulled straw vitrification method. Cryo-Letters, 19, 389–392.
63) Kuleshova, L., Gianaroli, L., Magli, C., Ferraretti, A. and Trounson, A. (1999): Birth following vitrification of a small number of human oocytes. Hum. Reprod., 14, 3077–3079.
64) Tominaga, K. and Hamada, Y. (2001): Gel-loading tip as container for vitrification of in vitro-produced bovine embryos. J. Reprod. Dev., 47, 267–273.
65) Papis, K., Shimizu, M. and Izaike, Y. (2000): Factors affecting the survivability of bovine oocytes vitrified in

droplets. Theriogenology, 54, 651–658.
66) Martino, A., Songsasen, N. and Leibo, S.P. (1996): Development into blastocysts of bovine oocytes cryopreserved by ultrarapidcooling. Biol. Reprod., 54, 1059–1069.
67) Lane, M. and Gardner, D.K. (2001): Vitrification of mouse oocytes using a nylon loop. Mol Reprod Dev., 58, 342–347.
68) Lane, M., Schoolcraft, W.B. and Gardner, D.K. (1999): Vitrification of mouse and human blastocysts using a novel cryoloop container-less technique. Fertil. Steril., 72, 1073–1078.
69) Hamawaki, A., Kuwayama, M. and Hamano, S. (1999): Minimum volume cooling method for bovine blastocyst vitrification. Theriogenology, 51, 165.
70) Kuwayama, M. and Kato, O. (2000): All round vitrification of human oocytes and embryos. J. Assist. Reprod. Gentic., 17, 477.