

Embryo and oocyte cryopreservation

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Embryo freezing

Mammalian embryos have been successfully frozen and stored since 1972, when Whittingham *et al.* obtained live mice after the transfer of frozen–thawed morulae (Whittingham *et al.*, 1972). Cryotechnology derived from rodents was applied to cattle industrially and represents, since 1980, >100 000 embryo transfers per year in the world.

The use of ovulation stimulation techniques to improve and simplify human IVF led to the recovery of large numbers of oocytes and consequently embryos. In order to limit the risks of multiple pregnancies arising from the transfer *in utero* of numerous embryos and to avoid the wastage of supernumerary embryos, the development of embryo cryopreservation was initiated for humans. The first human embryos were frozen in the late 1970s (Edwards and Steptoe, 1980) with the first pregnancy reported in 1983 in Australia (Trousion and Mohr, 1983). Then human embryo freezing spread rapidly and became an indispensable extension of IVF, with the help of optimized and simplified biological procedures, e.g. the use of propanediol and sucrose as cryoprotectants for pronucleate oocytes and early cleaved embryos (Lassalle *et al.*, 1985) and glycerol and sucrose for blastocysts (Cohen *et al.*, 1985; Fehilly *et al.*, 1985; Hartshorne *et al.*, 1991; Ménézo *et al.*, 1992).

Until now, protocols have mainly comprised a slow freezing down to -40°C and a rapid thawing. This is despite several attempts to vitrify both embryos and oocytes, with some success but no real advantage at present.

Embryo survival

The efficiency of a freezing programme is evaluated first on the morphological integrity of the embryo at thawing, second on its ability to further cleave *in vitro* and principally *in vivo*.

Early cleaved embryos are considered to have survived the freeze–thaw process when they keep at least half of their initial blastomeres intact after thawing and dilution of the cryoprotectants (survival rate = 50%). The survival rate, when

analysing the results of a freezing programme, is expressed as the percentage of surviving embryos among all frozen and thawed embryos. It usually represents at least 65% of thawed embryos (Figures 5.1–5.4).

Pronucleate oocytes are considered to have survived the freeze–thaw process when they appear intact after thawing, with a clear cytoplasm and no zona pellucida breaches, and when they are able to cleave *in vitro* during the next 24 h of culture.

Blastocyst survival is more difficult to appraise, considering the number of cells and their specialization. It is usual to recommend after thawing to transfer *in utero* only the morphologically normal blastocysts that have re-expanded after 3–4 h of recovery at 37°C in the culture medium.

Embryo freezing efficiency

The major aim of embryo cryopreservation is to provide further possibilities for conception in addition to those obtained through the initial cycle and fresh transfer. This goal is achieved with an increase in birth rate of 8% for the women who have embryos cryopreserved and by 5% for the overall programme (Mandelbaum, 1995). Embryo freezing has also contributed to lowering the risks of severe ovarian hyperstimulation by allowing the cancellation of the fresh transfer (Queenan *et al.*, 1997) and simplifying oocyte donation.

Whether freezing should be carried out at the blastocyst stage rather than at earlier stages is still a matter for debate. Blastocyst formation *in vitro* might enhance selection of the best embryos and allow a decrease in the number of embryos transferred.

Successful results have been reported with frozen–thawed blastocysts, grown in co-culture systems (Ménézo *et al.*, 1992). However, for reasons of safety, the use of heterologous cells has been replaced by a sequential media technology. Unfortunately, blastocysts obtained with those culture media seem to be less resistant to the freeze–thaw process than co-cultured blastocysts. Further studies are required to draw definite conclusions.

Until now, comparative analyses of pregnancies arising from fresh or frozen–thawed transfers have not found any differences, particularly with respect to fetal development, perinatal risk, obstetric outcome or the rate of congenital anomalies.

Oocyte freezing

Mature oocytes

In 1977, Whittingham obtained the first births from transfer of mice morulae, issued from mature oocytes frozen and stored

at -196°C (Whittingham, 1977). However, it was ~10 years until Chen reported the successful cryopreservation of human oocytes (Chen, 1986). Moreover, until 1997, very few births had arisen from human cryopreserved oocytes and only a handful of live babies had been reported in the literature.

Despite human oocytes being frozen and thawed according to techniques which had been applied successfully in other species (especially in rodents), survival rates remained low and fertilization rates were reduced after conventional IVF with a high incidence of polyploidy. Moreover, exposure to cryoprotective compounds and/or variations in temperature were claimed to have deleterious effects on oocyte structures, e.g. zona pellucida, cortical granules, spindle microtubules, cytoplasmic microfilaments and organelles.

Since 1994, fundamental studies began again in humans (Gook *et al.*, 1994), which showed good preservation of cell structures after freeze-thaw procedures carried out on mature oocytes. They were followed by a new set of clinical studies leading to the first new births for 10 years with embryos derived from cryopreserved mature oocytes (Porcu *et al.*, 1997).

Immature oocytes

Cryopreservation of immature oocytes, supposed to be less sensitive to cryo-injury, could be an advantage and could offer an opportunity to store the gametes of young patients requiring anti-cancer therapy for whom urgency of treatment may preclude the whole stimulation protocol used in mature oocyte collection.

Experimental studies on immature human oocytes were carried out after collection either in stimulated (Figures 5.5 and 5.6) or spontaneous cycles using mainly PROH and sucrose as cryoprotectants, applied according to the embryo freezing procedure. Nevertheless, no advantage of immature oocyte freezing could be drawn from these results, despite the birth of a normal female baby after cryopreservation of prophase I oocytes with subsequent in-vitro maturation (Tucker *et al.*, 1998). Improvements in the technique of oocyte in-vitro

maturation should precede the extension of immature oocyte freezing to reach satisfactory results.

References

- Chen, C. (1986) Pregnancy after human oocyte cryopreservation. *Lancet*, **i**, 884–886.
- Cohen, J., Simons, R.F., Edwards, R.G. *et al.* (1985) Pregnancies following the frozen storage of expanding human blastocysts. *J. In Vitro Fertil. Embryo Transfer*, **2**, 59–64.
- Edwards, R.G. and Steptoe, P.C. (1980) *Matter of Life*. Hutchinson, London, UK.
- Fehilly, C.B., Cohen, J., Simons, R.F. *et al.* (1985) Cryopreservation of cleaving embryos and expanded blastocysts in the human. A comparative study. *Fertil. Steril.*, **44**, 638–644.
- Gook, D.A., Osborn, S.M., Bourne, H. *et al.* (1994) Fertilization of human oocytes following cryopreservation; normal karyotypes and absence of stray chromosomes. *Hum. Reprod.*, **9**, 684–691.
- Hartshorne, G., Elder, K., Crow, S. *et al.* (1991) The influence of *in vitro* development upon post-thaw survival and implantation of cryopreserved human blastocysts. *Hum. Reprod.*, **6**, 136–141.
- Lassalle, B., Testart, J. and Renard, J.P. (1985) Human embryo features that influence the success of cryopreservation with the use of 1.2 propanediol. *Fertil. Steril.*, **44**, 645–651.
- Mandelbaum, J. (1995) Embryo freezing in humans: an overview. In Hedon B., Bringer J., Mares P. (eds), *Fertility and Sterility – A Current Overview. Proceedings of the 15th World Congress on Fertility and Sterility, 17–22 September 1995, Montpellier, France*. pp. 419–423.
- Ménézo, Y., Nicolle, B., Herbaut, N. *et al.* (1992) Freezing cocultured human blastocysts. *Fertil. Steril.*, **58**, 977–980.
- Porcu, E., Fabbri, R., Seracchioli, R. *et al.* (1997) Intracytoplasmic sperm injection of cryopreserved human oocytes. In *10th World Congress on In-Vitro Fertilization and Assisted Reproduction*. Monduzzi Eds, Bologna, Italy, pp. 1153–1157.
- Queenan, J.T., Veek, L.L., Toner, J.P. *et al.* (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. *Hum. Reprod.*, **12**, 1573–1576.
- Trounson, A.O. and Mohr, L. (1983) Human pregnancy following cryopreservation, thawing and transfer of an eight-cell embryo. *Nature*, **305**, 707–709.
- Tucker, M.J., Wright, G., Morton, P.C. *et al.* (1998) Birth after cryopreservation of immature oocytes with subsequent *in vitro* maturation. *Fertil. Steril.*, **70**, 578–579.
- Whittingham, D.G. (1977) Fertilization in-vitro and development to term of unfertilized mouse oocytes previously stored at -196°C . *J. Reprod. Fertil.*, **49**, 89–94.
- Whittingham, D.G., Leibo, S.P. and Mazur, P. (1972) Survival of mouse embryos, frozen to -196°C and -289°C . *Science*, **178**, 411–414.

Figure Legends

Figure 5.1

(5.1.1) A 5-cell frozen–thawed human embryo with one blastomere lysed after thawing. (5.1.2) The same embryo after removal of the lysed blastomere using an assisted hatching needle (courtesy of M.C.Magli, Bologna, Italy).

Figure 5.2

An 8-cell stage frozen–thawed embryo with two blastomeres lysed after thawing (courtesy of M.C.Magli, Bologna, Italy).

Figure 5.3

A 3 day-old frozen–thawed embryo with only one intact blastomere. Despite exceptional reported pregnancies after transferring 4-cell-stage embryos with only one surviving blastomere, this type of embryo has an extremely low viability and should not be used in transfers (courtesy of S.Kahraman, Ankara, Turkey).

Figure 5.4

A 6-cell embryo with all blastomeres lysed after thawing (courtesy of M.C.Magli, Bologna, Italy).

Figure 5.5

Human oocyte freezing. Chromosomes on the equatorial plate of the metaphase II spindle (stained with propidium iodide) in a human oocyte frozen at prophase I, thawed and matured *in vitro* for 24 h (courtesy of R.Levy, J.-F.Guérin, Lyon, France, and J.Mandelbaum, Paris, France).

Figure 5.6

Chromosome and spindle configuration of a human frozen–thawed oocyte (metaphase II). Normal configuration of the metaphase II spindle and chromosomes in a human oocyte frozen at prophase I, thawed and matured *in vitro* for 24 h (confocal scanning microscopy). The chromosomes are stained with propidium iodide (red); the spindle appears in green (anti- α -tubulin antibodies stained with rhodol green) (courtesy of R.Levy, J.-F.Guérin, Lyon, France, and J.Mandelbaum, Paris, France).

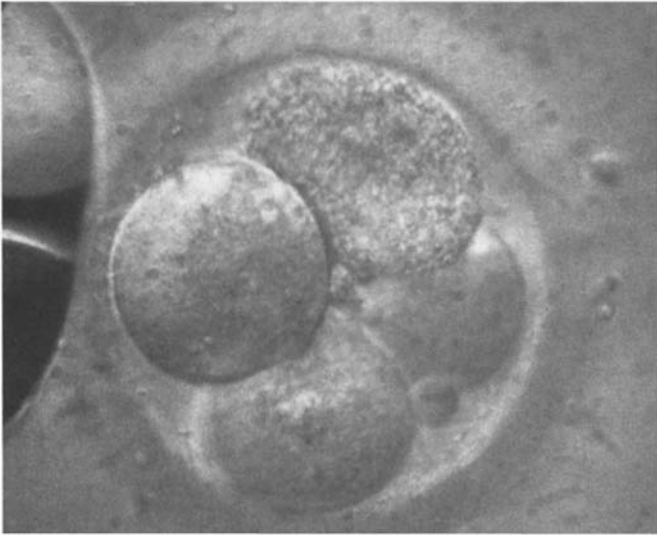


Figure 5.1.1

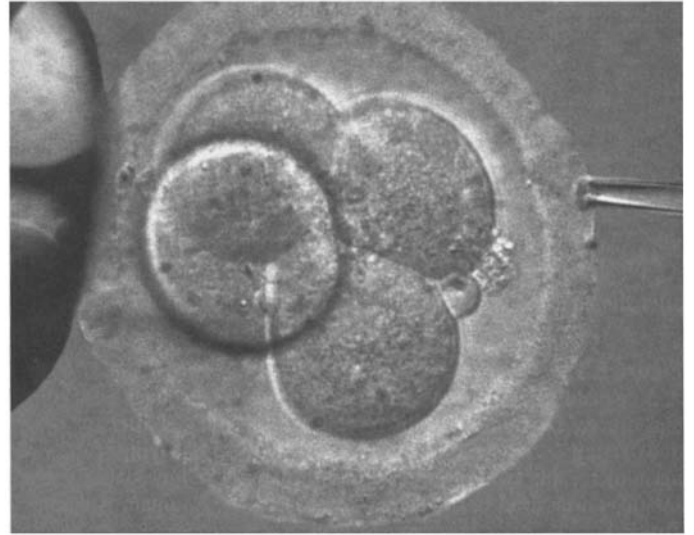


Figure 5.1.2

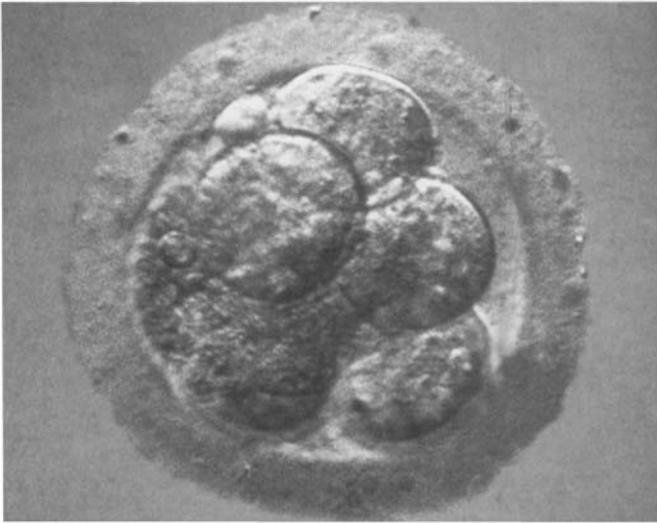


Figure 5.2



Figure 5.3

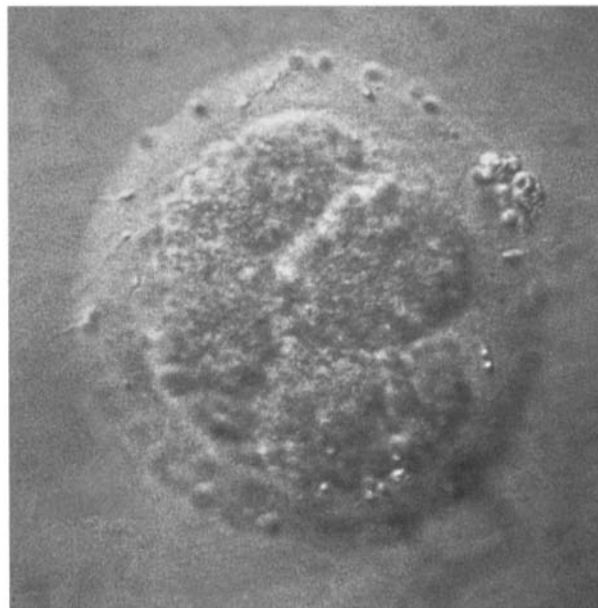


Figure 5.4

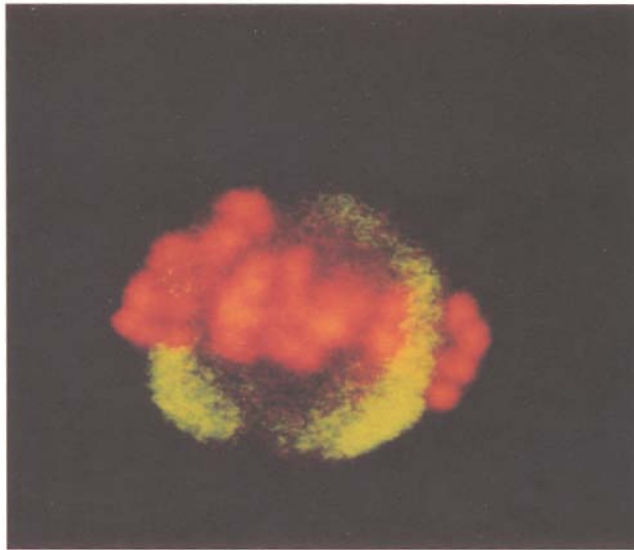


Figure 5.5

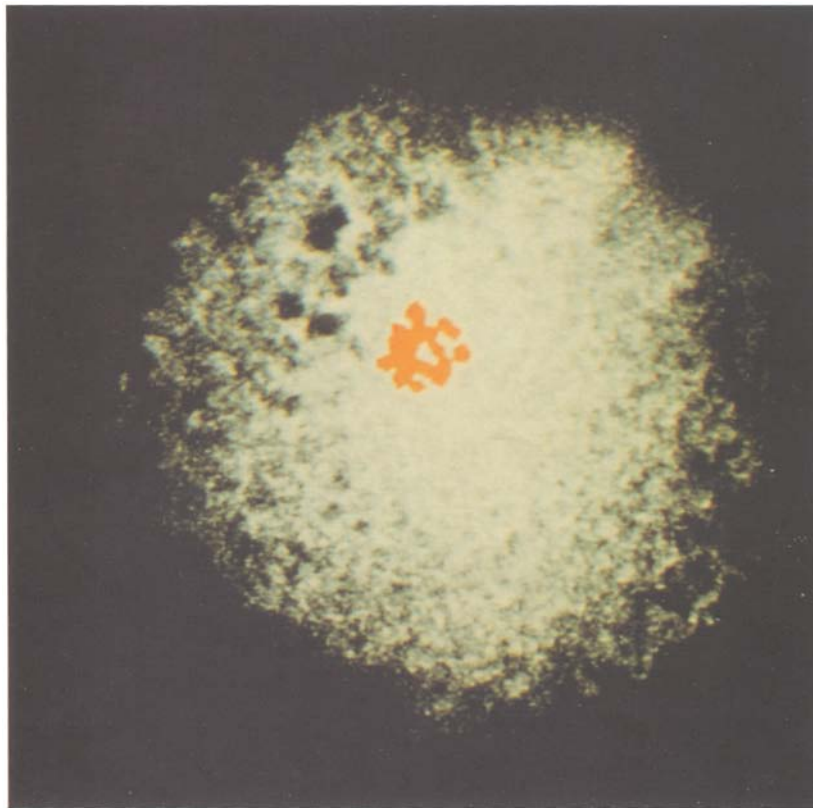


Figure 5.6