TECHNICAL NOTES

A Successful Technique for the Preservation of Rabbit Embryos

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Successful mammalian embryo freezing was first demonstrated in the mouse in 1972 by two independent groups (1, 2). Subsequently, live born or near term young have been produced from frozen embryos in two other laboratory species, the rat (3, 4) and the rabbit (5, 8). In 1965 it was stated that cells cooled below 0 C are subjected to three phenomena (9): 1. temperature drop, 2. ice crystal formation, and 3. solution effects. In light of these phenomena a few parameters become important: 1. the permeability of the cell membrane, 2. the surface to volume ratio of the cell, and hence, 3. the size of the cell. Therefore, a method that is optimal for the freezing of one cell type is not necessarily optimal for the freezing of another. In regard to the freezing of embryos, it has been shown (5) that while the 8 cell stage has been optimal for the mouse the morula state is better for the rabbit. Other investigators (8) supported this and demonstrated that late morulae were best in their experiments. A technique for successfully freezing, thawing and transferring rabbit embryos has been developed. Morula stage embryos were collected 64 hrs post coitus from superovulated female rabbits by flushing both oviducts and uterine horns with a tissue culture medium, Ham's F10-medium supplemented with 0.3 % Bovine Serum Albumin. Well developed, viable embryos were then transferred to freezing vials and a cryoprotectant, dimethylsulfoxide (DMSO) was added in several steps to bring its final concentration to 1.6 molar. To freeze the embryos seeding was done at –7 °C and the temperature was lowered slowly (either 0.5 °C/min or 0.1 °C/min) to –80 °C at which point the vials were transferred directly to liquid nitrogen (–196 °C). Thawing was done at 8 °C/min. After thawing, phosphate buffered saline was added in a stepwise manner to dilute the DMSO. The thawed embryos were then cultured at 37 °C. Transfer of the embryos was accomplished by laparotomizing a pseudopregnant doe using aseptic surgical technique and introducing the embryos into the fimbriated ends of the oviducts. A number of 101 transferred embryos resulted in 45 implantations and 34 live born young.

Our numbers are small but certain conclusions can be drawn. Superovulation is desirable and a comparison of our data with others (10) indicates the age of the doe may be a factor in maximizing the number of morulae obtained. The freezing technique is workable and results with rabbits compare favourably with work on mouse embryos at the Jackson Laboratory. Hybrid recipients should be used and considerable practice is necessary.

References

1. *Whittengham, D. G., S. P. Leibo & P. Mazur:* Survival of mouse embryos frozen to —196 C and —269 C. *Science* 1972; 178: 411—414.
2. *Wilmut, I.:* The effect of cooling rate, warming rate, crvoprotective agent and stage of development on survival of mouse embryos during freezing and thawing. *Life Sci.* 1972; 11: 1071—1079.
3. *Utsumi, K. & M. Yuhara:* Survival of frozen rat embryos, freezing and storage of rat blastocysts by dry ice alcohol. *Sci. Rep. Fac. Agr.* Okayama Univ. 1974; 44: 24—27.
4. *Whittingham, D. G.:* Survival of rat embryos after freezing and thawing. *J. Reprod. Fert.* 1975; 43: 575—578.
5. *Bank, H. & R. R. Maurer:* Survival of frozen rabbit embryos. *Exp. Cell Res.* 1974; 89: 188—196.

6. *Maurer, R. R. & J. K. Haseman:* Freezing morula stage rabbit embryos. *Biol. Reprod.* 1976; 14: 256—263.
7. *Whittingham, D. G. & C. E. Adams:* Low temperature preservation of arbbit embryos. *J. Reprod. Fert.* 1976; 47: 269—274.
8. *Tsunoda, Y. & T. Sugie:* Survival of rabbit eggs preserved in pastic straws in liquid nitrogen. *J. Reprod. Fert.* 1977; 49: 173—174.
9. *Mazur, P.:* The role of cell membranes in the freezing of yeast and other single cells. *Ann. NY Acad. Sci.* 1965; 125: 658—676.
10. *Kenelley, J. J. & R. H. Foote:* Superovulatory response of pre- and postpubertal rabbits to commercially available gonadotrophins. J. Reprod. Fert. 1965; *9*: 177—188.

Sammendrag ved O. Meyer

I 1972 lykkedes det for første gang for to uafhængige forskergrupper at nedfryse tidlige pattedyrfostre (1,2). Siden er der blevet produceret nyfødte fra rotte (3, 4) og kanin (5, 8) ud fra frosne embryoner. I 1965 blev det fastslået, at celler, der køles til under 0°C, er underkastet følgende tre faktorer (9): 1. Temperaturfald. 2. dannelse af iskrystaller og 3. ændrede opløselighedsforhold. Som følge af disse faktorers indflydelse vil der ske ændringer i en række parametre, bl. a. 1. Permeabiliteten af cellemembranen, 2. ændringer i forholdet mellem cellens overflade og volumen og således 3. størrelsen af cellen. En metode der synes optimal for nedfrysning af en celletype behøver ikke at være specielt velegnet for andre celletyper. Således er det vist, at nedfrysning af tidlige musefostre sker bedst ved »8-cellestadiet«, medens morulastadiet er bedst, når det drejer sig om tidlige kaninfostre (5). Andre forskere har i overensstemmelse hermed vist, at nedfrysning af tidlige kaninfostre sker bedst i det sene morulastadium (8). En anvendelig teknik til nedfrysning, optøning og overførsel af tidlige kaninfostre er blevet udviklet. Fostre på morulastadiet blev opsamlet 64 timer efter parring fra »superovulerende« kaniner ved at gennemskylle æggeledere og begge livmoderhorn med vækstmedium, Ham's Flo-medium tilsat 0,3 % Bovint Serum Albumin. Veludviklede, levedygtige fostre blev overført til »fryseampuller«, og et »frysebeskyttende« middel, dimethylsulfoxid (DMSO) blev tilsat i gradvis stigende koncentrationer op til 1,6 molar. Fostrene blev overført til ampuller med vækstmedium ved —7°C, og temperaturen blev langsomt sænket (enten 0,5°C/min. eller 1,0°C/min.) til ÷ 80°C, og på dette tidspunkt overførtes ampullerne direkte til flydende kvælstof (÷ 196°C). Optøning foregik ved 8°C/min. Efter optøningen blev en phosfatbuffer-saltopløsning tilsat i stigende koncentrationer til fortynding af DMSO. De optøede fostre blev dyrket ved 37°C. Fostrene blev overført til pseudodrægtige kaniner ved en regelret laparotomi. Placeringen af fostrene skete i æggeledernes fimrehårbeklædte del. 101 overførsler af tidlige fostre resulterede i 45 implantationer og 34 levende fødte.

På trods af det forholdsvis lille forsøgsmateriale kan følgende konklusioner drages. Superovulering er ønskværdig og en sammenligning af vores data med andre peger på, at hunkaninens alder kan være en betydende faktor for maximal »høst« af morulae (tidlige fostre i morulastadiet) (10). Fryseteknikken er anvendelig, og resultaterne er fuldt på højde med det tilsvarende arbejde med musefostre på Jackson Laboratory. Hybridrecipienter (evt. udavlede dyr) anbefales, og god øvelse er en nødvendighed.