

## Direct rehydration of in vitro fertilised bovine embryos after vitrification

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*Veterinary Record* (1994) 134, 276-277

VITRIFICATION is a valuable embryo freezing technique because it is simple, quick, economic (Rall and Fahy 1985), reliable and reasonably easy to apply under field conditions (Niemann 1991). Vitrification offers considerable promise for simplifying and improving the cryopreservation of cells because controlled-rate freezing equipment is not required and the potential injury associated with the formation of ice is eliminated (Rall 1987). The first successful vitrification of bovine embryos was achieved by Massip and others (1986). The survival of vitrified embryos is influenced by the type of cryoprotectant used and the exposure procedures (Yang and others 1992). Leibo (1989) suggested that embryos can be successfully vitrified in either glycerol-based or propylene glycol-based solutions. Sucrose and other carbohydrates like trehalose are effective in preserving the structural and functional integrity of membranes at low water activities (Massip and others 1987). Compact morulae seem to be the most suitable stage for vitrification, and the survival rate of expanded blastocysts can be increased up to 10-fold by using Massip solution and by equilibration of 4°C (Cseh and others 1992).

In the present study, in vitro fertilised embryos were cryopreserved in a simpler way by using direct rehydration in modified phosphate buffered saline (PBS) after vitrification.

Oocytes were aspirated from the ovaries collected from a local slaughterhouse and matured for 20 to 22 hours at 38.5°C in 5 per cent carbon dioxide in air. These matured oocytes were fertilised by in vitro capacitated sperm and cultured in vitro until blastocysts formed. The blastocysts were used for the vitrification experiment on days 8 and 9.

Three types of vitrification solutions were used. In the first type the embryos were kept in 10 per cent ethylene glycol for five minutes and then in 10 per cent ethylene glycol in 0.3M trehalose for five minutes. Finally, the embryos were kept in 40 per cent ethylene glycol in 0.3M trehalose for one minute. The first two media were at room temperature but the third was pre-cooled on ice. The embryos were placed in 0.25 ml straws which were immersed horizontally in liquid nitrogen after being held in the vapour for 20 seconds. The frozen embryos were thawed in a waterbath at 20 to 25°C. After thawing, the embryos were placed directly into the modified PBS at room temperature for five minutes and washed two or three times, and then placed in the culture medium. The second and third types of vitrification solution contained an additional 5 per cent and 10 per cent Percoll, respectively, in the final solutions. All the solutions were prepared with PBS supplemented with 0.3 per cent bovine serum albumin (w/v) and 50 mg/ml gentamicin.

Twenty embryos were used with each type of vitrification medium, and the results are shown in Table 1.

TABLE 1: Viability of bovine embryos following vitrification

Type of vitrification solution	Number of embryos	Survival after thawing (%)	Survival after 24 hours (%)	Number of hatched embryos (%)
1	20	16(80) <sup>a</sup>	10(50)	3(15)
2	20	13(65)	8(40)	4(20)
3	20	8(40) <sup>b</sup>	2(10)	-

<sup>a, b</sup> Within columns, means with different superscripts are significantly different ( $P < 0.01$ )

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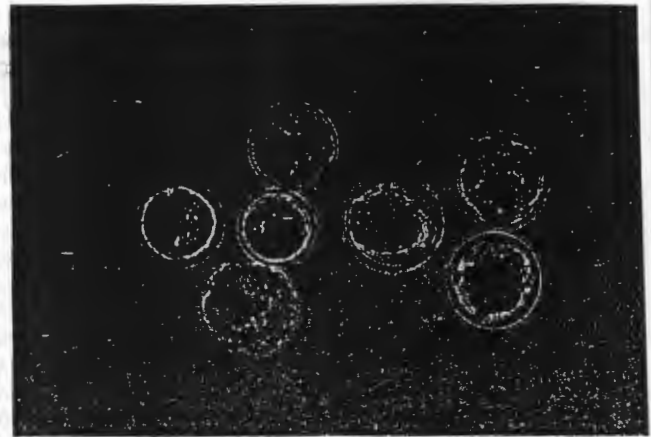


FIG 1: Day 8 and day 9 vitrified blastocysts after thawing (type 1)

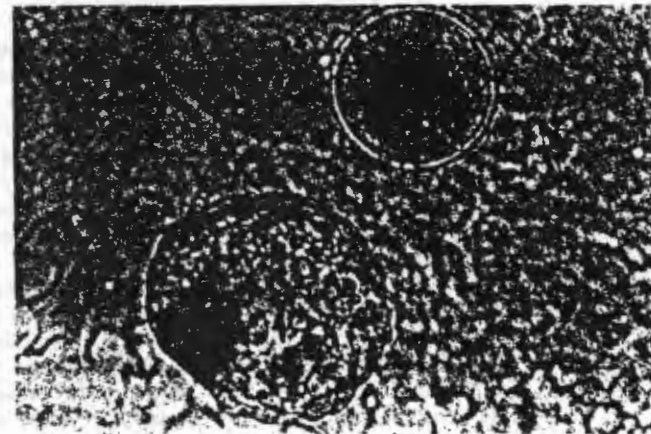


FIG 2: Hatched blastocyst after 48 hours of in vitro culture (type 1)

In the first five minutes the cryoprotective solutions used for equilibration contained 10 per cent ethylene glycol, and in the next five minutes they contained 10 per cent ethylene glycol with 0.3M trehalose. These two steps were done at room temperature. In the next step the embryos were put into solutions pre-cooled on ice and containing 40 per cent ethylene glycol with 0.3M trehalose for about one minute. During this gradual equilibration, substantial amounts of ethylene glycol entered the embryonic cells, and at the same time trehalose helped to dehydrate the embryonic cells, a process which is very important for successful vitrification. In the experiment in which 5 per cent Percoll was used the proportion of embryos which survived was similar, possibly because the Percoll helped the embryo to overcome cold shock, but when 10 per cent Percoll was used significantly fewer embryos survived, possibly because at this concentration Percoll was toxic to the embryo. The viability of the embryos was determined visually as the number which recovered from the dehydrated state (survival after thawing), the number of blastocysts which survived (survival after 24 hours) and the number of blastocysts which developed to hatched blastocysts (number of hatched embryos).

With the type 1 solution, 80 per cent of the blastocysts survived after thawing (Fig 1) and after 24 hours of culture the survival rate was 50 per cent. This result was lower than the value observed by Ishimori and others (1992) who used in vivo day 7 morulae, and a little lower than the value observed by Dobrinsky and others (1991) who used in vivo day 6 late morulae or early blastocyst stages, however, the survival rate was higher than that observed by Van Der Zwalmen and others (1989). After 48 hours of culture three (15 per cent) hatched blastocysts were obtained (Fig 2). The results obtained with the type 2 solution were not significantly different from those obtained with the type 1 and type 3 solutions, but the results obtained with the type 3 solution were significantly less satisfactory than those obtained with the type 1 solution.

