Direct rehydration of in vitro fertilised bovine embryos after vitrification

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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)[a]	10(50)	3(15)
2	20	13(65)	8(40)	4(20)
3	20	8(40)[b]	2(10)	–

a, b Within columns, means with different superscripts are significantly different ($P<0·01$)

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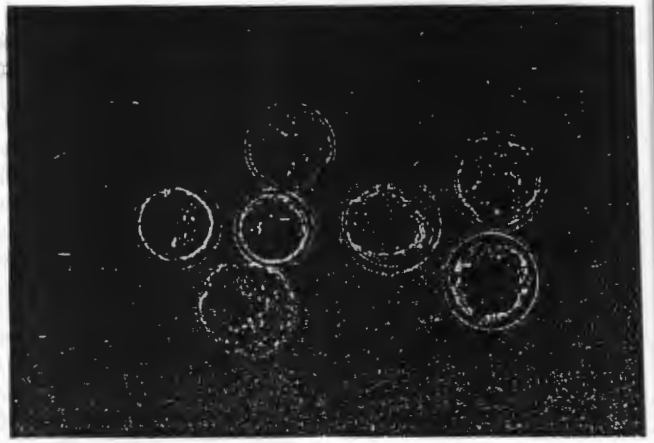

FIG 1: Day 8 and day 9 vitrified blastocysts after thawing (type 1)

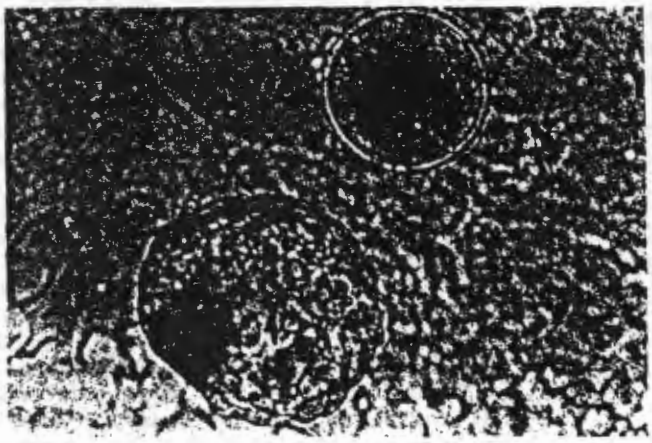

FIG 2: Hatched blastocyst after 48 hours of in vitro culture (type 1)

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 equlibration, 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.

These results suggest that the direct rehydration of in vitro fertilised bovine embryos after vitrification is useful. Most of the embryos survived the process of thawing, but their later development in the culture medium depended on the quality of the embryos. However, the direct rehydration in modified PBS made the procedure simpler and less time consuming.

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Fat absorption in female boxer dogs with undiagnosed hormonal alopecia

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CANINE hormonal alopecia may be associated with hyperadrenocorticism, hypothyroidism, growth hormone deficiency, imbalances of oestrogens, androgens and progesterone and diabetes mellitus (Muller and others 1989). In some cases, particularly of seasonal flank of ovariectomised female dogs (Scott 1990), despite extensive evaluation of the dog's hormonal status, the nature of the imbalance remains undetermined. Disturbances of fat metabolism associated with hyperadrenocorticism, hypothyroidism and diabetes mellitus result in profound alterations of the fat absorption curve (Simpson and van den Broek 1990, 1991). Imbalances of oestrogens, androgens and progesterone also influence fat metabolism (Kissebah and Schectman 1987) and may therefore be accompanied by alterations in the fat absorption curve. In this study quantitative fat absorption was evaluated in six female boxer dogs presented at the Royal (Dick) School of Veterinary Studies with undiagnosed hormonal alopecia.

Quantitative fat absorption was studied in two groups of dogs.

Group 1 was made up of 10 clinically normal dogs comprising seven neutered and three entire female animals aged between two and nine years (median age five years one month) and the following breeds: German shepherd dog (three); labrador retriever (two); springer spaniel (two); rough collie (one); Yorkshire terrier (one); crossbred (one).

Group 2 consisted of six boxers aged between two and seven years (median age five years four months). One of the boxers was an entire female, three had been spayed after the first season and two after the second season. All cases presented with a non-inflammatory, non-pruritic bilaterally symmetrical, hyperpigmented area of alopecia which had originated in the sublumbar region and in some cases had extended to the dorsal lumbar area or the lateral aspect of the thorax. The age at which the alopecia was first observed ranged between one and a half and six and a half years and, in the neutered animals, occurred six months to four years after ovariohysterectomy. In four cases there was a history of spontaneous hair regrowth which had occurred at different times of the year in each dog.

All the dogs were submitted to the following investigation: skin scrapings and biopsies were taken from affected sites, thyroid and adrenocortical function were assessed, respectively, by the thyroid-stimulating hormone response (Thoday 1990) and adrenocorticotrophic hormone or dexamethasone suppression tests (Feldman 1983). Plasma oestradiol, testosterone and progesterone concentrations were estimated and growth hormone status evaluated by xylazine stimulation (Lothrop 1989).

Quantitative fat absorption was determined using the method described by Simpson and van den Broek (1990). After a 12-hour fast each dog was fed 3 ml/kg corn oil. Venous blood samples (92 ml) were collected in anticoagulant-free Vacutainer tubes (Becton Dickinson) immediately before and 60, 120, 180 and 240 minutes after feeding corn oil. The serum was harvested and the triglyceride concentration determined with a Technicon 500 auto-analyser, using a commercial kit (Technicon).

The mean concentration of triglyceride in serum at each of the sampling times and the area under the curve were calculated for each group of dog. Differences between the two groups were compared by the Student's two sample *t* test using separate variance estimation for each group.

In the dogs with hormonal alopecia, no evidence of fungal infection or parasitic infestation was detected by microscopic examination of skin scrapings. Histopathological examination of skin biopsies revealed hyperkeratosis, increased pigmentation of basal cells, reduced numbers of hairs in the hair follicles, follicular keratosis and reduction in the size of sebaceous (four cases) and apocrine (three cases) glands. In all the dogs, evaluation of the hormonal status gave results within the normal range.

The number of normal dogs and dogs with undiagnosed hormonal alopecia, together with the mean, standard deviation and range of values at each sampling time are given in Table 1. The mean concentration of triglyceride in the serum of dogs with undiagnosed hormonal alopecia was significantly lower than that of

TABLE 1: Concentration (mmol/litre) of serum triglyceride and AUC for normal dogs and dogs with undiagnosed hormonal alopecia

Time (mins)	Parameter	Normal (n = 10)	Alopecia (n = 6)
0	Mean	0·56	0·49
	Range	0·38-0·77	0·38-0·62
	sd	0·10	0·10
60	Mean	0·91	0·55*
	Range	0·44-1·89	0·38-0·78
	sd	0·42	0·14
120	Mean	1·51	0·76***
	Range	0·62-2·26	0·50-0·98
	sd	0·48	0·17
180	Mean	1·90	0·98**
	Range	0·74-3·05	0·55-1·84
	sd	0·72	0·47
240	Mean	1·62	1·23
	Range	0·63-2·48	0·65-1·90
	sd	0·63	0·49
AUC	Mean	1·35	0·79**
	range	0·78-2·05	0·56-1·03
	sd	0·38	0·19

AUC Area under the curve
sd Standard deviation
Significant differences from normal are indicated by *P<0·05, **P<0·01, ***P<0·001

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