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# Successful vitrification of day-6 sheep embryos

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The aim of the experiments described here was to investigate cryopreservation of day-6 sheep embryos by vitrification methods in which the preliminary procedures can be performed at room temperature using VS1 (5.5 mol ethylene glycol $l^{-1}$ and 2.5 mol glycerol $l^{-1}$), VS11 (6.0 mol ethylene glycol $l^{-1}$ and 1.8 mol glycerol $l^{-1}$) and VS14 (5.5 mol ethylene glycol $l^{-1}$ and 1.0 mol sucrose $l^{-1}$). None of the day-6 sheep embryos vitrified with VS1 survived. Day-6 sheep embryos with the exception of blastocysts were vitrified with VS11 with no loss of viability *in vitro*. The viability of transferred day-6 embryos vitrified with VS11 was however extremely poor. Osmotic damage was avoided by initially exposing the embryos to one of four dilutions (20%, 30%, 40% and 50%) of VS11 for 5 min at 25°C and then vitrifying with the undiluted VS11. The highest survival (88.2%) *in vitro* was obtained when embryos were exposed to 30% VS11 before vitrification with the undiluted VS11. Survival of transferred embryos exposed to 30% VS11 and then vitrified with undiluted VS11 was 55% (16 of 29) for morulae and 62% (18 of 29) for blastocysts. The pregnancy rate for recipients that received two vitrified sheep embryos of these developmental stages per ewe was 79% (22 of 28). In a small study performed with VS14 the survival of day-6 sheep embryos vitrified with VS14 (in one-step) was 100% *in vitro* and 50% after transfer.

## Introduction

There have been two approaches to the cryopreservation of embryos. The first was slow freezing and, after the report of live births from frozen mouse embryos (Whittingham *et al.*, 1972), the technique has been applied to a number of other mammalian species including sheep (Willadsen *et al.*, 1976). The other approach is vitrification, which was first suggested by Luyet (1937), and successfully applied to mouse embryos by Rall (1987). Vitrification eliminates the formation of ice which can be lethal to embryos. An additional advantage of vitrification is that the very rapid freezing rate required to achieve vitrification, at concentrations of cryoprotectants that are not immediately toxic to embryos, can be achieved by plunging them into liquid nitrogen, thus eliminating the need for controlled-rate cooling apparatus. Sheep embryos have proven more sensitive than mouse embryos to the vitrification procedures and only moderate success has been achieved using solutions in which the major permeating cryoprotectants were glycerol and propylene glycol (Szell *et al.*, 1990; Schiewe *et al.*, 1991). After an extensive investigation of the vitrifying properties and embryotoxicity of a number of permeating cryoprotectants, Ali and Shelton (1993, in press) reported the successful vitrification of mouse embryos in vitrification solutions (VS) in which ethylene glycol was the major permeating cryoprotectant and using a procedure that was performed at room temperature (25°C). The solutions were VS1 (5.5 mol ethylene glycol $l^{-1}$ and 2.5 mol glycerol $l^{-1}$), VS11 (6.0 mol ethylene glycol $l^{-1}$ and 1.8 mol glycerol $l^{-1}$) and VS14 (5.5 mol ethylene glycol $l^{-1}$ and 1.0 mol sucrose $l^{-1}$). The experiments reported here were conducted to assess the viability of day-6 sheep embryos vitrified with VS1, VS11 and VS14.

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## Materials and Methods

### *Superovulation of donor ewes*

The oestrous cycles of Merino ewes were synchronized by the insertion of intravaginal sponges containing 40 mg fluges-tone acetate (Chrono-Gest, Intervet Australia Pty, Artarmon) for 12 days. Two days before removal of the sponges the ewes were superovulated by an i.m. injection of 12 mg FSH and 500 iu of pregnant mares' serum gonadotrophin.

Immediately after removal of the sponges, the ewes were joined with harnessed rams. Fertilization was ensured by carrying out intrauterine insemination by laparoscopy 36–48 h after sponge removal.

### *Collection of embryos*

The day-6 sheep embryos (morulae, early blastocysts and blastocysts) were recovered from the donors under general anaesthesia (Pentothal, Bomac Laboratories, NSW). Each uterine horn was flushed by injection of Hepes-buffered medium 199 (Flow Laboratories, Irvine) containing 5% fetal calf serum through the fimbrial end of the oviduct and its collection

through a Foley catheter (10FG 2.7 mm; Norta, Malaysia) inserted anterior to the body of the uterus. The embryos were pooled in droplets of Hepes-buffered synthetic oviduct fluid (HSOF), containing 20% sheep serum, under paraffin oil. HSOF was synthetic oviduct fluid (SOF; Tervit *et al.*, 1972) with 25 mmol sodium Hepes $l^{-1}$ and 4 mmol sodium bicarbonate $l^{-1}$. The pooled embryos were classified according to their stage of development and quality (Lindner and Wright, 1983) and those classified 'good' or 'excellent' were apportioned equally as appropriate for individual experiments.

*Exposure and vitrification of embryos*

Embryos were either exposed to VS in 100 μl drops under paraffin oil at 25°C without subsequent vitrification or were vitrified after exposure. Embryos to be vitrified were exposed to the vitrification solution as described above and within the allotted exposure time for each experiment they were loaded into 0.25 ml plastic insemination straws (IMV, L'Aigle). Each straw was prepared by filling it with VS and the plug end was heat sealed. With the VS-filled straw held horizontally, embryos (average of four per straw) were introduced with a fine pipette 35–40 mm from the open end of the straw which was then heat sealed. The sealed straw was then plunged horizontally into liquid nitrogen and held below the surface for 10–20 s (cooling rate approximately 2500°C $min^{-1}$; Rall, 1987). The straws containing vitrified embryos were stored in liquid nitrogen until use. Embryos that were not vitrified were not loaded into straws.

*Warming, dilution of VS and culture of embryos*

The embryos were warmed by directly immersing the vitrified straws in a water bath maintained at 25°C where they were held for 6–10 s (warming rate approximately 1000°C $min^{-1}$; Rall *et al.*, 1986). The contents of the straw were emptied into 1.0 ml of 1.0 mol sucrose $l^{-1}$ and stirred gently to facilitate mixing of the two solutions. The embryos were tranferred to a 100 μl drop of 1.0 mol sucrose $l^{-1}$ under paraffin oil at 25°C for 10 min and finally into HSOF at 25°C for 5 min before use in various experiments. The cryoprotectants in the embryos that were exposed to cryoprotectant but not vitrified were removed in a similar manner. The embryos were finally transferred to SOF and cultured up to the blastocyst or hatching blastocyst stages at 37°C in an atmosphere of 5% $CO_2$, 5% $O_2$ and 90% $N_2$. Untreated day-6 sheep embryos served as controls.

*Transfer of embryos*

Embryos vitrified with VS11 and VS14 were transferred after warming to recipient ewes in which the time of oestrus was controlled by the insertion of intravaginal sponges for 12 days and administration of 400 iu pregnant mares' serum gonadotrophin at the time of sponge withdrawal. Six days after oestrus, the ewes were sedated (Rompun, Bayer Australia Ltd, Botany) before transfer of embryos by laparoscopy. The exteriorized horn was punctured, and embryos (two per ewe) were introduced in a small amount of medium through a tom-cat catheter affixed to a 1 ml syringe.

Pregnancy was determined by real time ultrasonography at 30–37 days after the transfer of embryos. The pregnancies were confirmed by surgery on day 50 of pregnancy or the ewes were allowed to proceed to term.

*Statistical analysis*

Comparisons between groups were done by $\chi^2$ analysis except for group sizes of less than 25 when Fisher's exact test was used.

## Results

*Experiment 1: exposure or vitrification of day-6 sheep embryos*

The various developmental stages of day-6 sheep embryos were exposed to, or vitrified after exposure with, VS1, VS11 or VS14. Exposure was for 1–3 min (blastocysts were exposed for 1 min; early blastocysts for 2 min; morulae for 3 min) with VS1 and VS11 and for 2 min with VS14. After warming and dilution the embryos were cultured.

VS1 and VS11 were not toxic to day-6 embryos with the exception of VS1 to early blastocysts (Table 1). The survival of all developmental stages of day-6 sheep embryos was significantly reduced by vitrification with VS1. The survival of morulae and early blastocysts was not affected by vitrification with VS11 but the survival of blastocysts was significantly reduced. All developmental stages of day-6 sheep embryos were not affected (7 of 7) by vitrification with VS14 (not shown in Table 1).

*Experiment 2: vitrification of sheep blastocysts after partial equilibration*

In Expt 1 the survival of blastocysts was reduced by vitrification after exposure to VS11 for 1 min. The aim of Expt 2 was to test other exposure times for blastocysts. Sheep blastocysts, expanded blastocysts and collapsed blastocysts were subjected to partial equilibration in VS11 for 1, 3 or 5 min at 25°C and vitrified with VS11. The blastocysts were cultured after warming and dilution.

Although few embryos were used in the experiment, the results clearly suggest a decline in the rate of survival of sheep blastocysts and expanded blastocysts vitrified with VS11 when the duration of prior equilibration exceeded 1 min. This effect was not evident with collapsed blastocysts (Table 2).

*Experiment 3: dehydration of day-6 sheep embryos before vitrification*

The survival of all the collapsed blastocysts, albeit a small number, in Expt 2 suggested that removal of the blastocoelic fluid might be beneficial to survival of vitrified embryos. In Expt 3 the embryos were first dehydrated by exposure to 1 mol sucrose $l^{-1}$ for 3 min at 25°C. They were then partially equilibrated by exposure to VS11 for 1, 3 or 5 min, vitrified and treated as described above.

**Table 1.** Percentage survival *in vitro* of day-6 sheep embryos exposed* to or vitrified with VS1 and VS11

| Vitrification solution | Treatment | Stage of development: Blastocyst | Early blastocyst | Morula |
|---|---|---|---|---|
| VS1 | Control | 100 (6)** | 96.4 (28) | 59.0 (39) |
| | Exposed | 100 (4) | 64.3 (14)[a] | 59.5 (42) |
| | Vitrified | 11.1 (9)[b] | 24.2 (33)[c] | 26.8 (41)[b] |
| VS11 | Control | 88.9 (27) | 87.5 (24) | 71.7 (46) |
| | Exposed | 75.0 (20) | 80.0 (5) | 68.0 (20) |
| | Vitrified | 11.5 (26)[c] | 81.8 (22) | 85.7 (42) |

*Durations of exposure were 1 min for blastocysts, 2 min for early blastocysts and 3 min for morulae. **Numbers in parentheses are numbers of embryos in the group. [a]$P < 0.05$ compared with controls; [b]$P < 0.005$ compared with controls; [c]$P < 0.0001$ compared with controls.

**Table 2.** Percentage survival *in vitro* of day-6 sheep blastocysts equilibrated for 1, 3 or 5 min at 24°C and vitrified with VS11

| Stage of development | Duration (min) of equilibration with VS11: Untreated controls | 1 | 3 | 5 |
|---|---|---|---|---|
| Blastocysts | 87.5 (8)* | 50.0 (8) | 0 (8) | 12.5 (8)[a] |
| Expanded blastocysts | 100 (2) | 50.0 (2) | 0 (8) | 0 (2) |
| Collapsed blastocysts | — | — | 100 (2) | 100 (2) |

*Numbers in parentheses are numbers of embryos in the group. [a]$P < 0.05$ compared with controls.

After warming and culture, only one of 11 blastocysts survived *in vitro*. No expanded blastocysts (of 10), early blastocysts (of 16) or morulae (of 13) survived.

*Experiment 4: vitrification of day-6 sheep embryos after exposure to dilutions of VS11*

Sheep embryos in earlier experiments showed quite good survival *in vitro* after vitrification in VS11. However, observations on survival of embryos transferred to recipients after vitrification in VS11 were less encouraging. The possibility that a more gradual introduction to the high osmolarity of VS11 might be less traumatic was examined by exposing day-6 embryos for 5 min in 20, 30, 40 or 50% VS11 at 25°C, and then vitrifying with 100% VS11 (two-step method). The embryos were cultured after warming and VS dilution with sucrose.

There were no significant effects of exposure to any dilution of VS11 before vitrification on the survival of individual developmental stages (Table 3). When all the developmental stages were grouped together the survival of the embryos that were exposed to 20% and 50% VS11 for 5 min and then vitrified with 100% VS11 was significantly reduced ($P < 0.05$ and $< 0.005$, respectively). The survival of embryos was not affected when they were vitrified after exposure to 30% or 40% VS11. The survival rate in the latter two groups was 88.2% and 85.7%, respectively.

*Experiment 5: viability of vitrified day-6 sheep embryos when transferred to surrogates*

The viability *in vivo* of embryos vitrified in VS11 and VS14 was tested by transferring vitrified day-6 sheep embryos, after warming, to recipient ewes. In the first part of the experiment, embryos were vitrified in VS11 after direct exposure to 100% VS11 (1 min for blastocysts, 2 min for early blastocysts and 3 min for morulae) in one step. In the second part, embryos were vitrified after exposure to 30% VS11 for 5 min and to 100% VS11 for 1.5 min. In addition in this part of the experiment a small number of embryos were vitrified after 2 min exposure to VS14 in one step. The VS in embryos vitrified with VS11 (one-step) was removed by sucrose dilution or directly with HSOF medium (without sucrose dilution). In all other treatments, VS was removed by sucrose dilution.

The viability of embryos after transfer to recipients was poor when day-6 embryos were vitrified in one step with VS11 and was not affected by the method of dilution (Table 4).

**Table 3.** Percentage survival *in vitro* of day-6 sheep embryos equilibrated with various dilutions of VS11 at 25°C before vitrification with VS11

| Stage of development | Percentage dilution of VS11 for equilibration | | | | |
|---|---|---|---|---|---|
| | Untreated controls | 20 | 30 | 40 | 50 |
| Expanded blastocysts | 88.9 (9)* | 55.6 (9) | 81.8 (11) | 66.7 (9) | 33.3 (3) |
| Blastocysts | 100 (12) | 100 (7) | 90.9 (11) | 100 (10) | 66.7 (3) |
| Early blastocysts | 100 (6) | 33.3 (3) | 88.9 (9) | 100 (4) | 83.3 (6) |
| Morulae | 100 (5) | 100 (3) | 100 (3) | 80.0 (5) | 40.0 (5) |
| Total | 96.9 (32) | 72.7 (22)[a] | 88.2 (34) | 85.7 (28) | 58.8 (17)[b] |

*Numbers in parentheses are numbers of embryos in group. [a]$P < 0.05$ compared with untreated controls; [b]$P < 0.005$ compared with untreated controls.

**Table 4.** Survival of day-6 sheep embryos vitrified with VS11 and VS14 when transferred to recipient ewes

| Vitrification solution (method) | Developmental stage of embryos | Number of embryos | | Number of ewes | |
|---|---|---|---|---|---|
| | | Transferred | Survived (%) | Recipient | Pregnant (%) |
| VS11 (one-step)* | Blastocysts | 30 | 0 | 12 | 0 |
| | Early blastocysts | 60 | 1 (1.7) | 33 | 1 (3.0) |
| | Morulae | 68 | 7 (10.3) | 34 | 7 (20.6) |
| VS11 (two-step)** | Expanded blastocysts | 4 | 2 (50.0) | 2 | 1 (50.0) |
| | Blastocysts | 29 | 18 (62.1) | 14 | 11 (78.6) |
| | Early blastocysts | 10 | 1 (10.0) | 5 | 1 (20.0) |
| | Morulae | 29 | 16 (55.2) | 14 | 11 (78.6) |
| VS14 (one-step)** | Expanded blastocysts | 4 | 0 | 2 | 0 |
| | Blastocysts | 4 | 4 (100.0) | 2 | 2 (100.0) |
| | Morulae | 2 | 1 (50.0) | 1 | 1 (100.0) |

*Pooled results for dilution of VS with and without sucrose (1 mol $l^{-1}$). **Diluted with sucrose (1 mol $l^{-1}$).

The embryo survival and pregnancy rates for the different developmental stages that were exposed to 30% VS11 for 5 min and then vitrified with 100% VS11 (two-step) were: morulae 55% and 79%, blastocysts 62% and 79%, early blastocysts 10% and 20% and expanded blastocysts 50% and 50%, respectively.

The embryo survival and pregnancy rates of the small number of sheep morulae and blastocysts that were vitrified with VS14 were 50% and 100%, and 100% and 100%, respectively. None of the four expanded blastocysts that were vitrified with VS14 survived *in vivo*.

## Discussion

Studies on the vitrification of mouse embryos (Ali and Shelton, 1993) indicated that complete equilibration with cryoprotectant was unnecessary. It was suggested that in addition to cryoprotective action, a major role of the VS was to induce dehydration in embryos, thereby greatly increasing the intracellular solute concentrations to allow intracellular glass formation during cooling. The VS provides extracellular protection by forming extracellular glass during cooling. The same appears to be true for day-6 sheep embryos.

Day-6 sheep embryos were quite tolerant to VS1 but did not survive vitrification in this solution. No further investigations were therefore performed with VS1. Sheep early blastocysts and morulae can be vitrified with VS11 with no loss of viability *in vitro*. Previous studies (Ali, 1992; Ali and Shelton, 1993) performed on mouse preimplantation embryos suggested that the toxicity of VS1 and VS11 was probably positively related to the amount of glycerol present in the VS. VS11 has less gylcerol (1.8 mol $l^{-1}$) than VS1 (2.5 mol $l^{-1}$) and is less toxic. The studies in Expt 1 showed little difference in toxicity between VS1 and VS11 when day-6 sheep embryos were exposed to these solutions for 1–3 min, but there was a significant difference in survival after vitrification. It is not clear whether this is due to enhanced embryotoxicity of VS1 under conditions of vitrification or to inadequate cryoprotection by

VS1. Survival of blastocysts was severely reduced by vitrification. Valdez *et al.* (1990) reported a similar sensitivity of mouse blastocysts to vitrification. In our experiments there was no deleterious effect on early sheep blastocysts.

Experiments conducted to determine whether the lower viability of sheep blastocysts vitrified with VS11 was related to insufficient equilibration revealed that equilibration for 3 min or more was detrimental to their survival. However, the survival *in vitro* of collapsed blastocysts vitrified after equilibration for 3–5 min with VS11 suggested that the prior removal of the blastocoelic fluid could be beneficial and could enhance the survival of the vitrified blastocyst. Experiments to test this suggestion proved otherwise when the blastocoelic fluid was removed by challenging the blastocyst with 1 mol sucrose $l^{-1}$. Day-6 embryos of all developmental stages did not survive when they were subjected to dehydration with sucrose after which they were equilibrated for 1, 3 or 5 min and then vitrified with VS11. It is, however, obvious that the turgidity of the blastocysts is somehow related to higher sensitivity to the VS, possibly because they suffer greater osmotic trauma and damage than do non-turgid blastocysts when challenged with the VS. Turgid blastocysts may be more vulnerable than collapsed blastocysts because sudden challenge with a highly concentrated permeating solution may result in abrupt ultrarapid movement of a relatively large volume of fluid across the cell membrane. This may result in damage.

The reason for the poor survival *in vivo* of embryos vitrified in one step compared with the survival *in vitro* is not clear. Studies (Ali, 1992) have shown that viability of sheep morulae *in vitro* was not reduced by exposure to VS11 until 20 min at 25°C. Studies on volume changes in the embryo following exposure to VS (Ali, 1992) also appear to confirm this suggestion. The data presented here and elsewhere (Ali, 1992; Ali and Shelton, 1993) suggest that, in spite of survival *in vitro* of embryos vitrified in one step, the number of blastomeres that survive vitrification are insufficient or mainly consisted of, or gave rise to, trophoblastic cells, rendering the embryo incapable of establishing a viable pregnancy. Indeed the number of cells in vitrified (one-step) early sheep blastocysts ($n = 5$; mean = 59.8; SEM = 13.10) that were cultured for 48 h after warming were significantly lower ($P < 0.005$) than those in untreated early blastocysts that were similarly cultured ($n = 4$; mean = 138.0; SEM = 11.95). It was concluded that a significant number of blastomeres of day-6 sheep embryos were irreversibly damaged during the one-step vitrification procedure, possibly owing to excessive osmotic stress, with the result that subsequent viability was reduced *in vivo* but not *in vitro*.

The problem of osmotic damage to the blastomeres of day-6 sheep embryos when exposed to VS11 (which is 7.8 mol $l^{-1}$) was overcome by initially exposing the embryos to one of the various dilutions of VS11 ranging from 20 to 50% before vitrification with the undiluted (100%) VS11. Initial exposure to 30 and 40% VS11 followed by vitrification with the undiluted VS11 resulted in the highest *in vitro* survival rates which were comparable to that of untreated embryos. It is suggested that this regimen provided less harmful osmotic steps than initial treatment with 20 or 50% VS11.

Day-6 sheep embryos vitrified by the two-step procedure, particularly morulae and blastocysts, resulted in high embryo survival and pregnancy rates when transferred to recipients. The viability of early sheep blastocysts *in vivo* was poor with a 10% embryo survival and 20% pregnancy rate. However, the number of embryos at this stage was too small to be conclusive. Experiments with a larger sample size would be needed to show any real effect of developmental stage.

Survival rates of 30 to 60% have been reported after transfer of embryos frozen–thawed by conventional methods with a variety of cryoprotectants (Willadsen, 1977; Tervit and Goold, 1984; Heyman *et al.*, 1987). There have been few reports of successful vitrification of sheep embryos. McGinnis and Youngs (1990) reported survival of blastocysts *in vitro* after vitrification and Schiewe *et al.* (1991) reported two live offspring from transfer of seven embryos vitrified in 6.5 mol glycerol $l^{-1}$ and 6% BSA. Szell *et al.* (1990) obtained 11% (4 of 35) *in vivo* survival of morulae and 32% (6 of 19) for blastocysts and hatching blastocysts vitrified in a medium consisting of 25% glycerol and 25% propylene glycol after exposure at 4–12°C. Thus, results reported here, 62% (18 of 29) for blastocysts and 51% (37 of 72) for all developmental stages of day-6 sheep embryos, are similar to those reported from conventional freeze–thaw procedures, and superior to those in earlier reports on vitrification. Furthermore, exposure to the vitrification medium was at 25°C. It is worth noting that, although there was no concurrent comparison of freeze–thaw procedures in the experiment reported here, in a previous experiment in this laboratory (Shelton, 1992), survival of embryos frozen in 1.5 mol ethylene glycol or glycerol $l^{-1}$ and transferred one per recipient was 45.2%. The data presented here and elsewhere (Ali and Shelton 1993, in press) strongly suggest that the use of ethylene glycol as the major permeating cryoprotectant in the vitrification medium is the reason for the greater success rate. Tervit and Goold (1984) and Heyman *et al.* (1987) remarked on the advantages of ethylene glycol in conventional procedures.

The results suggest that VS14 can be used to vitrify day-6 sheep embryos by a one-step procedure with little loss of viability. VS14 consists of 5.5 mol ethylene glycol $l^{-1}$ and 1.0 mol sucrose $l^{-1}$, whereas VS11 consists of 6.0 mol ethylene glycol $l^{-1}$ and 1.8 mol glycerol $l^{-1}$. Neither 5.5 mol ethylene glycol nor 1.0 mol sucrose $l^{-1}$ will vitrify on its own, but the combination will vitrify. Furthermore these concentrations of ethylene glycol and sucrose alone or in combination are non-toxic to embryos (Ali, 1992; Szell and Shelton, 1986). VS14 is potentially a very good vitrification agent for mammalian embryos and warrants further investigation.

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