Cloning of bovine embryos from vitrified donor blastomeres

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The use of cryopreserved *in vitro* produced bovine embryos as nuclear transfer donors was assessed. Day 4 or 5 morulae were vitrified and warmed using the open pulled straw method and used as donors for nuclear transfer. Although the proportion of morulae and blastocysts that developed from nuclear transfer embryos derived from day 5 vitrified embryos did not differ from that derived from fresh embryos (16.7 and 24.3%, respectively), development to blastocysts was reduced when vitrified donor cells were used (8.3 and 19.1%, respectively). Likewise, development to morulae and blastocysts was not different between nuclear transfer embryos derived from day 4 vitrified embryos allowed to recover for 24 h, and day 5 vitrified embryos allowed to recover for 1–2 h (27.7 and 15.6%, respectively), but the development to blastocysts was reduced when day 5 vitrified donor cells were used (23.2 and 10.0%, respectively). However, in nuclear transfer embryos derived from either day 4 vitrified or day 5 fresh donors, no differences were observed in development rates to morulae and blastocysts (34.3 and 36.3%, respectively) or to blastocysts alone (20.2 and 18.1%, respectively). Nor were there differences in the development rates of fresh or day 4 or day 5 vitrified *in vitro* produced (non-nuclear transfer) embryos (47.9, 51.0 and 35.5% developing to blastocysts at day 7, respectively). *In vitro* produced embryos and nuclear transfer embryos derived from day 4 vitrified or day 5 fresh donors were transferred to recipients at morula or blastocyst stage at day 6 or 7. The pregnancy rates were similar in both groups of nuclear transfer embryos, but higher in the control group consisting of *in vitro* produced embryos (47, 42 and 67%, respectively). In conclusion, if vitrified donor embryos are allowed to recover for 24 h after warming, their use in nuclear transfer results in similar efficiencies to those achieved with fresh embryos.

Introduction

Fundamental biological questions of development and differentiation can be resolved by nuclear transfer, where the nucleus of an oocyte or zygote is replaced with the nucleus of a developmentally more advanced cell. This method has also been used to produce offspring in livestock species (Willadsen, 1986; Prather *et al.*, 1987). In the majority of bovine nuclear transfer experiments, the donor embryos are used fresh without cryopreservation. Early studies in bovine embryo cloning used mainly *in vivo*-derived embryos, flushed from superovulated and inseminated donor animals on the day of nuclear transfer (Willadsen *et al.*, 1991). Improved *in vitro* techniques have since facilitated the production of donor embryos for nuclear transfer purposes (Zakhartchenko *et al.*, 1996). However, there is a need for a long-term storage of *in vivo* and *in vitro* produced embryos. The successful production of large numbers of identical

offspring from a single embryo relies on recycling nuclear transfer embryos as donor cells for subsequent rounds of nuclear transfer. However, this approach is limited by several factors. The inability to control the progression of the recycling process once it has commenced and the lack of abattoir-derived oocytes on weekends limits the number of recycling rounds that can be performed. In addition, full use of a large number of clones derived from one original donor embryo becomes increasingly difficult as the number of recycling rounds increases. The ability to cryopreserve nuclear transfer donor embryos successfully would remove these limitations in recycling and enable the recycling to be fully controlled, thus improving the overall efficiency of cloning techniques.

Few studies have compared the developmental capacity of nuclear transfer embryos derived from cryopreserved or non-cryopreserved embryos. Westhusin *et al.* (1991) and Yang *et al.* (1993) compared fresh and frozen-thawed *in vivo*-derived bovine embryos as donors in nuclear transfer, and observed no differences in fusion or embryonic development rates. However, Heyman *et al.* (1994) observed a significant reduction in embryonic development after using vitrified-

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warmed *in vivo*-derived bovine embryos as compared with non-vitrified controls. Likewise, Ushijima *et al.* (1996) reported reduced development rates when *in vitro* produced bovine donor embryos had been delipated and vitrified at the one-cell stage and used as cell donors for nuclear transfer at the 8–16-cell stage.

A novel vitrification technique called the open pulled straw technique (OPS) has been developed and successfully applied to embryos of various developmental stages and species (Vajta *et al.*, 1998). Preliminary reports from our laboratory (Peura *et al.*, 1998a) as well as from the laboratory where this technique was developed (Vajta *et al.*, 1997a) indicate that this technique can be applied to nuclear transfer donor embryos. In the present study, the effects of the developmental stage of donor embryos at the time of vitrification and the recovery time after warming on the nuclear transfer outcome were evaluated, to establish optimal procedures for using vitrified donor embryos in bovine embryo cloning.

Materials and Methods

Production of embryos and oocytes

Bovine oocytes and embryos were produced *in vitro* as described by Peura *et al.* (1998b). Briefly, immature oocytes were aspirated from abattoir-derived ovaries and matured in groups of 50 in 800 μ l TCM-199 (Gibco BRL, Grand Island, NY) containing 10% fetal calf serum (FCS; Gibco) and 0.01 iu ml^{-1} bovine LH and bovine FSH (NOBL Laboratories, Sioux Center, IA) at 39°C in an atmosphere of 5% CO_2 in air. Donor embryos were produced from oocytes fertilized after 22–24 h maturation in groups of ten in 50 μ l fertilization drops with frozen-thawed Percoll (Sigma Chemical Co., St Louis, MO) separated bull spermatozoa at a concentration of 2×10^6 spermatozoa ml^{-1} , at 39°C in an atmosphere of 5% CO_2 in air. Twenty-four hours after fertilization, cumulus cells were removed by vortexing and the putative zygotes were cultured for 3 days at 39°C in an atmosphere of 5% CO_2 , 7% O_2 and 88% N_2 in 500 μ l modified synthetic oviductal fluid (mSOF) (Edwards *et al.*, 1997) containing Eagle's non-essential amino acids (ICN Biomedicals Inc., Aurora, OH) and 8 mg BSA ml^{-1} (Sigma) in four-well culture dishes (Nunc, Roskilde). The embryos were then cultured in mSOF containing Eagle's essential and non-essential amino acids (ICN) and 8 mg BSA ml^{-1} for a further 3 days. Handling of immature oocytes was done at room temperature, whereas all subsequent stages of oocyte and embryo handling were performed on warm stages (LEC Instruments, Scoresby, Vic) at 38°C.

Embryo vitrification and warming

In vitro produced embryos were vitrified either at day 4 or day 5 (day 0 = day of fertilization). In either case, only embryos at the expected stage of cleavage and development were vitrified according to the method of Vajta *et al.* (1997a). Briefly, embryos were first washed in a bench medium consisting of TCM-199, 25 mmol Hepes l^{-1} (Sigma) and 20%

FCS, followed by 3 min incubation in the bench medium containing 7.5% (v/v) dimethylsulfoxide (DMSO) (Sigma) and 7.5% (v/v) ethylene glycol (Sigma). The embryos were then incubated for 25 s in the bench medium containing 0.6 mol sucrose l^{-1} (Sigma), 16.5% (v/v) DMSO and 16.5% (v/v) ethylene glycol, during which time they were loaded into thin pulled straws by capillary action, in approximately 2 μ l medium, followed by immediate plunging into liquid nitrogen. The straws were prepared from 0.25 ml insemination straws (IMV, L'Aigle) by heating them above a coiled heating plate and pulling gently to achieve long, narrow straws. These were then cut with a razor blade to yield straws approximately 9 cm long and 0.8 mm outer diameter at the narrow end. For storage, straws were stored in loosely closed 15 ml conical tubes in liquid nitrogen.

Embryos were removed one straw at a time from liquid nitrogen and warmed in air for 2–3 s before submerging the narrow end of the straw into the first well of a four-well Nunc plate containing 1.2 ml bench medium with 0.3 mol sucrose l^{-1} . The other end of the straw was covered by a fingertip, causing the embryos to expel from the straw into the medium, where they were incubated for 5 min. The embryos were then transferred to the second well containing bench medium with 0.15 mol sucrose l^{-1} for another 5 min incubation, and then incubated twice, 5 min each, in the third and fourth well in the bench medium without sucrose. Embryos were then cultured in mSOF culture medium containing Eagle's essential and non-essential amino acids and 8 mg BSA ml^{-1} .

Nuclear transfer and embryo culture

Recipient oocytes were enucleated as described by Peura *et al.* (1998b). Briefly, cumulus cells were removed by vortexing and mature oocytes were selected according to the presence of an extruded first polar body. After agglutinating polar bodies to oocytes with phytohaemagglutinin (ICN) incubation and removing zonae pellucidae with Pronase (Sigma), oocytes were bisected unequally with a metal blade by free hand. The smaller portions of the oocytes (approximately 40% of the total oocyte volume) still attached to the polar body were discarded and the remaining, chromatin-free cytoplasts were used for nuclear transfer. This method routinely results in >95% enucleation efficiency, as demonstrated by chromatin staining with the fluorochrome Hoechst 33342 (Sigma) and examination under ultraviolet illumination. Cytoplasts were subsequently activated in 38 mmol Ca^{2+} -ionophore l^{-1} (Sigma) in protein-free TCM-199 for 10 min followed by a 4 h incubation in 2 mmol 6-dimethylaminopurine (6-DMAP) l^{-1} (Sigma) in TCM-199 plus 10% FCS, after which they were used for cell fusions.

Donor embryo disaggregations and nuclear transfers were performed as described by Peura *et al.* (1998b). Briefly, after removal of zona pellucida with pronase, donor embryos were disaggregated into individual blastomeres in cytochalasin B-supplemented medium. Cytoplasts and blastomeres were fused in mannitol fusion medium in an electrofusion chamber containing parallel wires with a

diameter of 0.5 mm and a separation of 0.5 mm. For each nuclear transfer embryo, two cytoplasts and one blastomere were aligned with an AC pulse of 8–10 V and 500–600 kHz for 5–10 s, then fused with a single DC pulse of 100 μ s and an amplitude of 600 V cm^{-1} . Reconstituted embryos were cultured individually in 20 μ l drops. For the first 3 days, the embryos were cultured in mSOF supplemented with Eagle's non-essential amino acids and 20 mg BSA ml^{-1} and, after that, in mSOF supplemented with Eagle's essential and non-essential amino acids and 20 mg BSA ml^{-1} . Embryo cleavage and developmental stage were recorded 3, 6 and 7 days after fusion. The number of blastocyst cells was determined by placing embryos in an ethanol fixative containing 25 mg Hoechst 33342 ml^{-1} at 4°C in the dark overnight. Embryos were then mounted in glycerol (Sigma) on a microscope slide, pressed down with a coverslip and the fluorescent nuclei were counted under ultraviolet light using an excitation filter of 340–380 nm.

Experiment 1

Fresh and vitrified then warmed day 5 embryos were assessed as nuclear transfer donors. Briefly, some morulae at day 5 of culture were vitrified, warmed immediately and returned to the culture for another 3 h, while the rest of the embryos were left in culture. Embryos from both groups were used subsequently as donors for nuclear transfer. Recipient cytoplasts were produced earlier the same day and used immediately after the end of DMAP incubation. Reconstituted embryos from both groups were cultured under identical conditions until day 7, and fusion, cleavage and development rates were recorded.

Experiment 2

Embryos were vitrified and warmed either at day 4 or day 5, allowed to recover for 24 or 1–2 h, respectively, after warming and assessed as nuclear transfer donors. Briefly, precompaction stage morulae at day 4 of culture were selected and divided into two groups. Embryos in one group were vitrified, immediately warmed, changed into fresh medium and cultured for an additional 24 h. Embryos in the other group were changed into fresh medium, cultured for an additional 24 h, after which the morphologically normal embryos (average 90% of embryos left to culture) were vitrified, immediately warmed and returned to culture for 1–2 h. Embryos from both groups were used as nuclear donors for nuclear transfer, using the recipient cytoplasts prepared earlier on that day. Reconstituted embryos were cultured under identical conditions until day 7. Fusion, cleavage and development rates to morulae and blastocysts, and total cell numbers of blastocysts were recorded.

Experiment 3

Embryo development after vitrification and warming either at day 4 or day 5 was examined to assess whether

developmental stage affected the survival of vitrified embryos. Precompaction stage morulae at day 4 of culture were selected and divided into three groups. Embryos in the control group were changed into fresh medium and cultured until day 7. Embryos in the second group were vitrified, immediately warmed, changed into fresh medium and cultured until day 7. Embryos in the third group were changed into fresh medium, cultured for an additional 24 h, after which the morphologically normal embryos were vitrified, immediately warmed and returned to culture until day 7. Development rates to morulae and blastocysts for all groups as well as blastocyst total cell numbers were recorded.

Experiment 4

Nuclear transfer embryos were produced using the best protocol as demonstrated in Expts 1 and 2 and transferred to recipients to evaluate the viability of nuclear transfer embryos derived from vitrified donors. As controls, *in vitro* produced embryos and nuclear transfer embryos derived from fresh donor embryos were also transferred. At day 4 after fertilization, a group of *in vitro* produced precompaction stage morulae were vitrified and immediately warmed, whereas the rest of the embryos were left intact. After a further 24 h in culture, embryos of both groups were used as donors for nuclear transfer. The resulting reconstituted embryos were cultured for 6 or 7 days and transferred to recipients. *In vitro* control embryos were produced separately, cultured for 7 days and transferred to the same group of recipients. Embryos were loaded in each insemination straw in Hepes-buffered mSOF medium containing 16 mg BSA ml^{-1} and transferred to recipients within 4 h of loading, during which time straws were kept at 37°C. Fifteen recipients received nuclear transfer embryos derived from fresh donors, fourteen receiving three and one receiving four embryos. Nuclear transfer embryos derived from vitrified donors were transferred to a total of 12 recipients, 11 receiving three and one receiving four embryos. Control embryos were transferred to a total of six recipients, each receiving two embryos. Recipients for embryo transfer were synchronized with an intravaginal progesterone-releasing device (CIDR®, InterAg, Hamilton) inserted for 8 days, with a prostaglandin i.m. injection (Estruplan®, Parnell, Sydney) given 24 h before CIDR removal. Non-surgical embryo transfers into the uterine horn ipsilateral to the corpus luteum were performed 6–7 days after standing oestrus was first observed. Pregnancy was determined by ultrasonography 40 days after embryo transfer. The number of fetuses in each recipient, as identified by ultrasonographic examination, was recorded.

Statistical analyses

Fusion rates were determined as the proportion of successfully fused, reconstituted embryos from the number of attempted fusions. All subsequent cleavage and embryo development rates were calculated from the total number

Fig. 1. Fusion and development rates of bovine nuclear transfer embryos derived from *in vitro* produced day 5 fresh (□) or vitrified (■) donor embryos. *Difference between columns significant ($P < 0.05$). Numbers of embryos fused or cultured are in parentheses.

Fig. 2. Fusion and development rates of bovine nuclear transfer embryos derived from *in vitro* produced day 4 (□) or day 5 (■) vitrified donor embryos, allowed to recover after vitrification for 24 or 1 h, respectively. *Difference between columns significant ($P < 0.05$). Numbers of embryos fused or cultured are in parentheses.

of successfully fused embryos. Fusion, cleavage and development rates between the experimental groups in all four experiments were compared by unpaired *t* tests (Expts 1, 2 and 4) or by the analysis of variance (ANOVA; Expt 3) after arcsin transformation of the original percentage data. Differences in embryonic cell numbers were compared without the data transformation. In both cases, the statistical package Graphpad Instat™ (GraphPad Software) was used.

Results

Experiment 1

A total of 14 fresh and 11 vitrified day 5 donor embryos were used for nuclear transfer in five replicate experiments. Mean cell numbers obtained from the embryos after disaggregation were 27.6 ± 2.5 from fresh and 34.0 ± 3.3 from vitrified (mean $\pm$ SEM). Fusion and cleavage rates did not differ significantly from each other between the two groups. Although there were no differences in the development rates to morulae and blastocysts at day 7 (24.3 and 16.7% for fresh and vitrified donors, respectively) (Fig. 1), development to blastocysts was significantly better ($P = 0.0221$) for nuclear transfer embryos derived from fresh donors as opposed to vitrified donors (19.1 versus 8.3%, respectively).

Experiment 2

A total of 54 donor embryos were vitrified and warmed in five replicate experiments. Some embryos (< 10% of total) were lost during the warming procedures, and further losses occurred as some embryos did not recover from the procedures but degenerated soon after warming (20–30% of total). After disaggregation, since embryos yielding < 20 blastomeres were rejected (as is routinely done with fresh donors), only 24 embryos were eventually used for nuclear

transfer after disaggregation. There were no differences in the numbers of blastomeres obtained from embryos vitrified at day 4 compared with those vitrified at day 5 (25.3 ± 2.6 and 27.1 ± 2.8 , respectively). Again, fusion and cleavage rates did not differ significantly between the two groups (Fig. 2). Differences were neither observed in development rates to morulae or blastocysts at day 7 (27.7 and 15.6%, with day 4 and day 5 vitrified donors, respectively) (Fig. 2). However, development to blastocysts was significantly better ($P = 0.0290$) in nuclear transfer embryos derived from day 4 vitrified donors than it was in day 5 vitrified donors (23.2 versus 10.0%, respectively). Total cell numbers of nuclear transfer blastocysts were not significantly different between the two groups ($P = 0.2944$). A mean ($\pm$ SEM) of 87.2 ± 7.9 and 97.5 ± 5.6 cells were identified in blastocysts derived from day 4 ($n = 18$) and day 5 ($n = 8$) vitrified donors, respectively.

Experiment 3

More than 1500 oocytes were fertilized and cultured in five separate replicates to produce 623 precompaction stage day 4 embryos. The overall cleavage rate was 73.1% (1150/1573). Only grade 1 quality embryos on each day (day 4 and day 5) were vitrified, resulting in only 56% of the embryos selected initially being vitrified at day 5. The recovery rate of embryos after warming was 84%. Owing to this loss in recovery, subsequent embryonic development rates are expressed for all groups as actual development rates (%_{cult}), on the basis of the development of embryos cultured from each group. Development is also expressed as corrected development rate (%_{corr}), on the basis of the number of embryos initially selected for each group. Although day 5-vitrified embryos had the highest actual development rates, compared with day 4-vitrified and control embryos (75.0, 59.1 and 63.3% to morulae and blastocysts, and 59.1, 51.0 and 47.9% to blastocysts,

Table 1. Development rates of *in vitro* produced bovine embryos after vitrification–warming at day 4 or 5 after insemination (control: day 4 non-vitrified embryos)

Group	Number of embryos			Morulae + blastocysts			Blastocysts		
	Selected ^a	Vitrified	Cultured	<i>n</i>	% _{cult}	% _{corr}	<i>n</i>	% _{cult}	% _{corr}
Control	165	n.a.	165	104	63.3 ^b	63.3 ^b	79	47.9 ^b	47.9 ^b
Vitrified at day 4	174	174	149	88	59.1 ^b	59.1 ^b	76	51.0 ^b	51.0 ^b
Vitrified at day 5	178	107	88	66	75.0 ^b	45.1 ^c	52	59.1 ^b	35.5 ^b

^aAll embryos for the experimental groups were selected at day 4 after fertilization.

%_{cult}, percentage of the actual number of cultured embryos developing to morulae or blastocysts; %_{corr}, corrected percentage of embryos selected to the experimental group developing to morulae or blastocysts.

^{b,c}Values with different superscripts within a column differ significantly ($P < 0.05$).

respectively), they also had the lowest corrected development rate (45.1, 59.1 and 63.3% to morulae and blastocysts, and 35.5, 51.0 and 47.9% to blastocysts, respectively). No differences among groups were observed in actual development rates or, in development to blastocysts, in corrected development rates (Table 1). However, the difference in corrected development rates to morulae and blastocysts was significantly lower in day 5-vitrified donors ($P = 0.0236$). Total cell numbers (mean $\pm$ SEM) of the blastocysts were 95.1 ± 6.1 in the control group ($n = 29$), 99.2 ± 4.7 ($n = 45$) in the day 4-vitrified group and 98.7 ± 9.1 ($n = 24$) in the day 5-vitrified group. These cell numbers were not significantly different among groups.

Experiment 4

In vitro development rates of nuclear transfer embryos derived from day 4-vitrified and day 5 fresh donor embryos are shown (Fig. 3). As in Expts 1 and 2, there were no differences in blastomere numbers obtained after the disaggregation of embryos vitrified at day 4 and cultured overnight when compared with day 5 fresh embryos (35.8 ± 3.0 and 27.1 ± 3.0 , respectively). No statistically significant differences were observed in fusion or cleavage rates or in development rates to morulae and blastocysts (34.3 and 36.3% for vitrified and fresh donors, respectively) or to blastocysts (20.2 and 18.1% for vitrified and fresh donors, respectively) at day 6. The development rates were only recorded up to day 6 as, in two out of three replicates, the embryos were transferred into recipients at day 6. The pregnancy rates achieved are shown (Table 2).

Overall, from the pooled results of Expts 1, 2 and 4, these numbers of blastomeres (mean $\pm$ SEM) were obtained from disaggregated donor embryos: 32.1 ± 2.1 from fresh, 26.4 ± 2.0 from day 4-vitrified and 30.7 ± 2.3 from day 5-vitrified donors. These numbers were not significantly different among the three groups.

Discussion

These experiments indicate that, when appropriate vitrification and warming procedures are used, there are no significant differences in the developmental potential of

Fig. 3. Fusion and development rates of bovine nuclear transfer embryos derived from *in vitro* produced donor embryos, vitrified at day 4 and allowed to recover after vitrification for 24 h (□), or fresh embryos used at day 5 (■). Differences between columns were not significant ($P < 0.05$). Numbers of embryos fused or cultured are in parentheses.

bovine nuclear transfer embryos derived from *in vitro* produced fresh or cryopreserved donor embryos. Although development rate to blastocysts was decreased when vitrified donor embryos were used for nuclear transfer shortly after warming, this deleterious effect could be overcome by allowing a longer recovery period before use. The advantages of this approach are twofold. First, the embryos subjected to extensive cryodamage can be easily identified and discarded at the end of prolonged culture period according to their morphology. Second, embryos only slightly affected by the vitrification procedure will benefit from the prolonged recovery period. Vajta *et al.* (1997b) observed serious subcellular injuries in vitrified bovine blastocysts when viewed by electron microscopy. In addition to severe overall degeneration of some cells, ultrastructural damage was observed in mitochondria and in intracellular contacts, but this damage was restored progressively over 24 h. However, in the case of nuclear transfer, recovery of intracellular organelles and intracellular contacts is not essential because the recipient ooplasm provides all the cytoplasmic components needed. Therefore, the improved development may reflect the identification and avoidance of donor cells containing seriously cryodamaged nuclei.

Table 2. Day 40 pregnancy rates of *in vitro* produced and nuclear transfer bovine embryos derived from vitrified or fresh donor embryos

Group	Number of transferred embryos/ number of recipients	Number of fetuses ^a	Number of recipients pregnant
IVP control embryos	12/6	5 (42%)	4 (67%)
NT-embryos from fresh donors	46/15	14 (30%)	7 (47%)
NT-embryos from vitrified donors	38/12	7 (18%)	5 (42%)

^aAt least this many fetuses observed by ultrasonography.

IVP, *in vitro* produced; NT, nuclear transfer.

The cryopreservation method may play a role in comparison of fresh and cryopreserved morulae as donors in nuclear transfer. Westhusin *et al.* (1991) and Yang *et al.* (1993) found no detrimental effect of cryopreservation of day 5–6 *in vivo* embryos for nuclear transfer outcomes, whereas Heyman *et al.* (1994) found reduced development after cryopreservation of day 5.5 *in vivo* embryos. The difference between these results could be explained by the different cryopreserving techniques used. Traditional freezing by slow-cooling methods was used by Westhusin *et al.* (1991) and Yang *et al.* (1993) and vitrification was used by Heyman *et al.* (1994). Slow-cooling techniques for freezing late morula and blastocyst stage bovine embryos have been modified over the years, whereas vitrification techniques are still relatively new in embryo cryopreservation. However, slow-cooling techniques have not been successful when used to cryopreserve early preimplantation stage embryos (Pollard and Leibo, 1994). The high lipid content and high volume to surface ratio of bovine early preimplantation stage embryos renders them susceptible to cryoinjury induced by ice crystal formation and osmotic damage, both of which occur during freezing by slow-cooling. Besides the developmental stage, the origin of the embryo (*in vivo* or *in vitro*) affects its ability to survive freezing (Leibo *et al.*, 1996). The embryos used for cryopreservation in the current study were day 4 and 5 *in vitro* produced embryos at early pre-compaction and compacted morula stages. Previous investigations revealed that these embryos do not survive freezing by traditional slow-cooling techniques (T. Peura and I. Lewis, unpublished). Delval *et al.* (1998) used a different vitrification method for *in vitro* produced day 6 bovine embryos, which were subsequently used as donors for nuclear transfer. Although no comparison was made with non-vitrified donor embryos, the reported development rates after nuclear transfer were quite good (20.5% blastocysts from reconstituted embryos). The novel vitrification method used throughout this study has been used successfully for cryopreservation of embryos previously deemed to be extremely sensitive to cryoinjury, such as pig embryos and bovine oocytes (Vajta *et al.*, 1998). The major advantage of the OPS is the very high cooling and warming rate achieved because of the novel design of the micro-straws (20 000°C min⁻¹ as compared with 2500°C min⁻¹ with traditional 0.25 ml mini-straws).

The origin of the donor embryo may also be an important factor in defining the developmental potential after nuclear transfer. However, the comparison of nuclear transfer embryos derived from *in vivo* and *in vitro* donor embryos

reveals some discrepancies among different studies. Superior development of *in vivo*-derived nuclear transfer embryos has been reported by Yang *et al.* (1993) and Clement-Sengewald *et al.* (1992). Heyman *et al.* (1994) and Zakhartchenko *et al.* (1996) found no differences in the developmental potential of *in vivo* compared with *in vitro* derived nuclear transfer embryos. In the present study, all donor embryos were produced *in vitro*, yielding rates of *in vitro* development comparable with those of other studies. Development rates to morulae and blastocysts in the present study varied from 24 to 36% in fresh day 5 donors, from 28 to 34% in day 4 vitrified donors, and from 16 to 17% in vitrified day 5 donors. The present results support those of Booth *et al.* (1998), who observed no differences between *in vitro* developmental potential of nuclear transfer embryos derived from fresh or vitrified donors (40.1 versus 33.7% blastocysts, respectively).

Vajta *et al.* (1998) found impaired embryo development when embryos were vitrified at day 1 and day 2, but no differences from day 3 to day 7. The results of the present study confirm this. No differences were found in the developmental potential of *in vitro* produced embryos after vitrification at either day 4 or day 5 after fertilization. The number of blastomeres obtained from donor embryos was not significantly affected by vitrification, even though day 4-vitrified embryo groups yielded a slightly smaller number of blastomeres than day 5-vitrified embryos and day 5 fresh control embryos (means of 26.4, 30.7 and 32.1 blastomeres, respectively). Although this difference was not statistically significant, it is possible that the trend observed results from the interruption and subsequent retardation of development by vitrification. Yang *et al.* (1993) reported reduced blastomere recovery rates from frozen embryos when compared with fresh embryos (from 90% recovery rate to 60%), but a similar effect was not observed in the present study. Hardly any cell lysis was observed in vitrified embryos at disaggregation.

The percentage of recipients pregnant at day 40 did not differ between nuclear transfer embryos derived from fresh or vitrified donor embryos but, in both groups, it was lower than *in vitro* control embryos (47, 42 and 67%, respectively) (Table 2). Initially, quite high fetal survival rates were obtained from control and fresh donor groups as compared with vitrified donor group (at least 42, 30 and 18% of transferred embryos developing into fetuses, respectively). Ten of sixteen pregnant recipients were diagnosed as carrying multiple fetuses, either two or three per recipient, but some of these multiple pregnancies in the fresh donor

group were subsequently lost between days 40 and 110. This is not surprising, as low pregnancy rates and high pregnancy losses are common with nuclear transfer embryos (Yang *et al.*, 1993). In addition, increased fetal losses have been reported to result from uterine overcrowding when more than one fetus is present in the same uterine horn (Hanrahan 1983), as was the case in the present study. In view of the present results, the number of embryos transferred to recipients may need to be reduced to improve the chances of the development of early fetuses to live offspring.

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