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Comparison of the Efficacy of Conventional Slow Freezing and Rapid Cryopreservation Methods for Bovine Embryos

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Day 7 bovine morulae and early blastocysts were randomly assigned to one of four cryopreservation methods: (i) a modified conventional controlled slow freezing and stepwise dilution after thawing; and three methods which enable direct transfer of the embryo into the recipient upon thawing: (ii) conventional controlled slow freezing and a modification of a one-step procedure, (iii) vitrification with 6.5 *M* glycerol plus 6% BSA (w/v), and (iv) vitrification with 25% glycerol (v/v) and 25% propanediol (v/v). In a comparative *in vitro* study, the percentage of grade 1 and 2 embryos developing into expanded blastocysts in culture for cryopreservation methods 1–4 were, respectively, 53% (29/55), 33% (20/61), 44% (26/59), and 51% (17/33). Method 2 yielded a significantly lower survival rate than methods 1 ($P < 0.1$) and 4 ($P < 0.05$) and was excluded from a subsequent test of *in vivo* development. Pregnancy rates (Day 60) after transfer of embryos cryopreserved by methods 1, 3, and 4 were, respectively, 59% (20/34), 43% (17/40), and 24% (5/21). Method 4 yielded a significantly lower pregnancy rate than method 1 ($P < 0.05$). Method 3, however, did not yield a statistically different pregnancy rate ($P > 0.1$) when compared to method 1. Method 3 has considerable promise in providing a successful method for the cryopreservation of bovine embryos that (i) reduces the time required for equilibration and cooling, (ii) provides for simple and rapid one-step dilution of cryoprotectant after thawing, and (iii) enables more embryos to be thawed and transferred per unit time.

In bovine embryo transfer practice, controlled slow freezing, is routinely used for cryopreservation of Day 7 embryos. Reports of pregnancy rates for frozen–thawed grade 1 and 2 embryos usually range from 50 to 60% (7, 14). Considerable progress has been made over the past two decades in simplifying and improving nearly every step of the cryopreservation process for use in routine embryo transfer practice (19). The introduction of the use of straws as freezing containers by Tsunoda and Sagai (31) was of great importance, since it is essential for methods that include in-straw dilution and rapid cooling. Another great improvement resulted from the development of new procedures for removing cryoprotectants after thawing. The most common dilution method is a stepwise reduction in the concentration of cryoprotectant in the embryo suspension. This approach is time consuming (20–60 min) and limits the number of embryos that can be thawed and transferred per time unit.

In the past decade, cryopreservation methods have been developed for bovine embryos that enable direct transfer of the embryo into the recipient after thawing in a manner analogous to AI in cattle. Leibo and Renard (8, 9, 24) have described conventional freezing procedures which allow

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the removal of glycerol from thawed embryos within the freezing container (plastic insemination straw). These so-called "one-step" procedures require a sucrose diluent (0.3–1 *M*) to be placed in the straw container along with the embryo in a separate drop of saline containing glycerol. Premature mixing is prevented by the placement of small air bubbles between the solutions. This approach has been reported to yield an overall pregnancy rate of 30 to 60% for Day 7 bovine embryos (2, 4, 10, 24, 30).

A third improvement resulted from the development of a new cryopreservation method called vitrification which usually includes the use of a one-step dilution procedure (12, 20). One important cryobiological benefit of vitrification is that no ice forms in the embryo suspension during the period of cooling to −196°C, storage, and warming. This eliminates potential damage due to the formation of ice. A practical advantage of vitrification is a reduction in the time required for equilibration and cooling to −196°C from about 90 min for conventional freezing to less than 25 min. Reports of pregnancy rates after vitrification and one-step dilution range from 40 to 55% (12, 19).

Since experimental conditions vary greatly among previous papers of alternative cryopreservation methods, reported pregnancy rates cannot easily be compared. We have used an experimental design in which comparison of pregnancy rates between different cryopreservation methods is possible. Post-thaw survival rates are compared after conventional slow freezing and stepwise dilution to three methods which enable direct transfer of the embryo into the recipient upon thawing. These procedures include method 1, a modified slow freezing/stepwise dilution method; method 2, a modification of the one-step freezing procedure of Leibo (10); method 3, vitrification by the procedure of Rall (18, 19); and method 4, vitrification by a modification of Massip *et al.'s* procedure (12).

MATERIALS AND METHODS

Embryos. In experiment 1 (at Rio Vista International, San Antonio, TX, in 1986), evaluating the vitrification procedure of Rall (18, 19), cross-bred *Bos taurus* × *Bos inducus* beef cattle served as donor, sire, and recipient females. In experiment 2 (The Netherlands), Holstein dairy cattle served as donor, sire, and recipient females. Embryos were collected from superovulated cows by standard nonsurgical flushing of the uterus on Day 7 of the estrous cycle. Embryos were washed in PB1 (33) and classified as to their embryonic stage and grade.

In experiment 1, embryos were held in PB1 at 20–25°C for 1–3 h prior to cryopreservation. Embryos at the morula to expanded blastocyst stage with quality grades of excellent (1), good (2), or fair (3) were vitrified.

In experiment 2, embryos were held in PB1 at 20–25°C for 1–8 h prior to cryopreservation. Morulae and early blastocysts graded as 1–3 were randomly assigned to one of four cryopreservation treatment groups: (i) a modified conventional controlled slow freezing, (ii) conventional controlled slow freezing and a modification of the one-step dilution (10), (iii) vitrification and one-step dilution by the method of (19), or (iv) a modification of (12). Published procedures were modified as described below.

Cryoprotectant solutions. All cryoprotectant solutions consisted of PB1 to which glycerol was added at a concentration of 1.2, 1.5, 6.5 *M* and 25% (v/v), respectively, for methods 1–4. The vitrification solutions of methods 3 (solution VS3a) and 4 (Massip's VS) were further supplemented with, respectively, bovine serum albumin (6%, w/v) and 1,2-propanediol (25%, v/v). The cryoprotectant solutions of methods 2 (1.5 *M* glycerol) and 3 (VS3a) were prepared by

methods which maintained the salts concentration at the same molar concentration as that in PB1 (13, 17). All other cryoprotectant solutions were prepared by adding the appropriate number of moles of cryoprotectants to PB1, a procedure that maintains the salts concentration at the same molal concentration as that in PB1.

Controlled freezing. Embryos were frozen in methods 1 and 2 as follows. First, embryos were transferred into the appropriate cryoprotectant solution and held for 10–20 min at room temperature (20–25°C). Then embryos were loaded into a 91-mm straw (Z484, IMV, L'Aigle, France; method 1), a 0.25-ml insemination straw (A201, IMV; method 2, *in vitro* comparison), and in a 0.25-ml ET straw (ZA481, IMV; method 2, *in vivo* comparison) as shown in Fig. 1. Straws were sealed either with a plastic rod (ZA144, IMV, method 1) or by heat sealing (method 2). Straws were then placed vertically into the alcohol bath chamber of a FTS Biocool controlled rate freezing machine (Stone Ridge, NY) that was precooled to about −7°C and immediately seeded. Ten minutes later, the alcohol bath was cooled at 0.5 (method 1) or 0.4°C/min (method 2) to −35°C. After 10 min at −35°C, the straws were immersed and stored in liquid nitrogen (−196°C).

Vitrification. Embryos vitrified in experiment 1 and by method 3 were treated as described by Rall (18, 19). Briefly, embryos were rinsed in PB1 containing BSA (6%, w/v) and placed into PB1 containing glycerol (1.625 *M*) and BSA (6% BSA, w/v) for 10–20 min at room temperature (20–25°C). Then, embryos were rinsed in PB1 containing glycerol (4.2 *M*) and BSA (6%, w/v) for 1–1.25 min before transfer into the solution VS3a column in a preloaded straw (Fig. 1; A201, IMV, experiment 1 and *in vitro* comparison; ZA481, IMV, *in vivo*

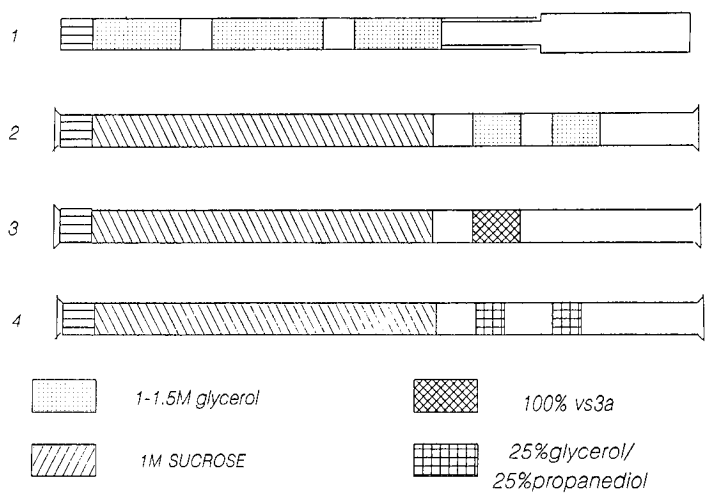

FIG. 1. Configuration of solutions within straws for cryopreservation methods 1–4. Straws were prepared by aspirating the appropriate sequence of cryoprotectant solutions (two 1.0-cm columns of 1.5 *M* glycerol in method 2 and two 0.5-cm columns of 25% glycerol/25% propanediol in method 4), sucrose diluent (7.5-cm column), and air into 0.25-ml transparent plastic straws (methods 1, 2, and 4). Each column of liquid was separated by air bubbles 0.5–1 cm long. Embryos were aspirated into the straw along with the second column of cryoprotectant solution in method 1. In method 3, the sucrose diluent and VS3a columns were loaded into the straws using 27-gauge, 1.5-in. needles attached to a 1-ml syringe, so that an air bubble 5 mm long with dry walls separated the two columns (Rall, 1992). In methods 2–4, embryos were transferred into preloaded straws. (See text for details).

comparison) and the straw was heat sealed. One minute after the embryo was transferred into solution VS3a, the straw was placed in cold nitrogen vapor (−170°C, 2 or 3 min) and then immersed and stored in liquid nitrogen (−196°C). Embryos vitrified by method 4 were treated by a modification of the procedures of Massip *et al.* (12). Briefly, embryos were transferred into PB1 containing glycerol (10%, v/v) and 1,2-propanediol (20%, v/v) for 10–15 min at room temperature. Then, embryos were transferred into a column of the final vitrification solution in a preloaded straw precooled to 4°C (Fig. 1; A201, IMV, experiment 1 and *in vitro* comparison; ZA481, IMV, *in vivo* comparison) and the straw was heat sealed. Thirty to 60 s later, the straw was placed in cold nitrogen vapor (−170°C, 2 or 3 min) and then immersed and stored in liquid nitrogen (−196°C). In methods 2–4, a 0.5-ml plastic insemination straw (A101, IMV) was slip fitted over the plugged end of each 0.25-ml straw and served as a handle (Leibo, 1984). Straws were stored in plastic goblets (PA004, IMV) attached to aluminum canes, the 0.25-ml straw below.

Warming and dilution. After a storage period of 1 day to 8 months, straws were warmed rapidly in air of 20–25°C for 10 s and then water of 35°C (method 1) or 20°C (methods 2–4) until all ice had disappeared (5–10 s). Then, embryos in method 1 straws were expelled and diluted in petri dishes using three equimolar step reductions of glycerol in PB1 containing 0.3 *M* sucrose (5 min/step). Embryos were then washed in PB1. Embryos in methods 2–4 straws were diluted by a modification of Leibo's procedure (10). Briefly, thawed straws were held at the plugged end and shaken to mix the cryoprotectant and sucrose diluent columns and yield single columns of air and liquid. Straws were placed vertically (plug-end down) in 36°C water for 3 min and then 20°C for about 1 min. Straws were then held at the end opposite to the plug, shaken as described above, and placed vertically (plug-end up) in 20°C water for 3–5 min. Finally, the heat seals were removed by cutting with scissors.

Assessment of embryo viability. Post-thaw *in vitro* survival rates were defined as the percentage of thawed embryos developing to expanded or hatching blastocysts during culture. In experiment 1, embryos were cultured for 72 h in 10-μl drops of CMRL-1066 media (Gibco, Grand Island, NY) supplemented with 1% BSA under silicone oil. In experiment 2, embryos were cultured individually for 96 h in 250-μl drops of Bavister's TALP culture medium (1) under paraffin oil. In both cases culture dishes were incubated at 38.5°C in a humidified atmosphere of 5% O_2, 5% CO_2, and 90% N_2.

Post-thaw *in vivo* development of embryos in experiment 1 was defined as the percentage of thawed embryos that established a 90 Day pregnancy following nonsurgical transfer to recipient cows or heifers. In experiment 1, the estrous cycle of recipient cows was monitored by observing estrous behavior during natural cycles. Cows exhibiting estrus and a well-formed corpus luteum by rectal palpation on Day 7 received a vitrified embryo that was thawed, diluted, and transferred by placing the straw container used for vitrification into a embryo transfer (ET) gun. All transfers were performed by the same person.

In experiment 2, post-thaw *in vivo* development was defined as the percentage of thawed embryos that established a 60-day pregnancy following nonsurgical transfer to recipient heifers. Only embryos cryopreserved by methods 1, 3, and 4 were evaluated. In method 1, diluted embryos were loaded into a 0.25-ml plastic straw (ZA481, IMV). In methods 3 and 4, embryos were not recovered from the straws used for vitrification, thawing, and dilution. Straws were loaded into an ET gun. Recipient females were synchronized using a SynchroMate B device (Crestar, Intervet,

Boxmeer, the Netherlands). Only those heifers exhibiting post-treatment estrus and a well-formed corpus luteum at palpation on Day 7 were used for transfer. All transfers were performed by the same person.

In both *in vivo* studies, the embryo was deposited in the horn ipsilateral to the ovary on which the corpus luteum was found on Day 7 of the recipient's cycle. Pregnancies were confirmed by rectal palpation on Day 90 (experiment 1) or Day 60 (experiment 2).

Data in experiment 1 were analyzed by χ^2 using Yate's correction for continuity. Data in experiment 2 were analyzed using logistical ANOVA using SAS procedure CATMOD (25). In the latter, survival rates of cryopreservation treatment groups are automatically adjusted for differences between cryopreservation treatment groups in the number of embryos for each statistically significant factor. For example, differences in the number of grade 1 and 2 embryos assigned to cryopreservation treatment groups were compensated for in comparisons of survival rates between cryopreservation methods. The respective models for analysis by logistical ANOVA *in vitro* and *in vivo* survival were:

$$\text{percentage expanded blastocysts}_{ijklm} = \mu + \text{cryopreservation group}_i + \text{embryo grade}_j + \text{embryo stage}_k + \text{time interval between collection and cryopreservation}_l + e_{ijklm}$$

$$\text{pregnancy rate}_{ijklmn} = \mu + \text{cryopreservation group}_i + \text{embryo grade}_j + \text{embryo stage}_k + \text{recipient quality}_l + \text{time interval between collection and cryopreservation}_m + e_{ijklmn},$$

where the model factors are (i) cryopreservation group = 1–4 for the *in vitro* study and 1, 3, and 4 for the *in vivo* study; (ii) embryo grade = 1–3 for the *in vitro* study and 1 or 2 for the *in vivo* study; (iii) embryo stage = morulae or early blastocysts; (iv) recipient quality = 6 or 7—a subjective score assigned by the ET technician for, respectively, fair or good recipients based on rectal palpation of corpus luteum and other ovarian structure; and (v) time interval between collection and cryopreservation = 0–2, 2–4, 4–6, or 6–8 h.

RESULTS

Experiment 1. The *in vitro* developmental capacity of a total of 52 grade 1–3 Day 7 bovine embryos at the morula to expanded blastocyst stage was determined following vitrification in solution VS3a (Table 1). Most thawed and diluted embryos developed to the expanded blastocyst stage (88%) and initiated hatching (73%) during, respectively, the initial 36 and 48 h of culture. No effect of initial embryo grade was found on the ability of vitrified embryos to develop *in vitro* to expanded blastocysts (*P*

TABLE 1
In Vitro Development of Bovine Embryos Following Vitrification in Solution VS3a, Rapid Warming, and in Straw Dilution (Experiment 1)

Embryo grade	No. of Embryos		
		Developing to vitrified expanded blastocysts (%)	Developing to hatching blastocysts (%)
Excellent (1)	12	11 (92)*	8 (67)*,†
Good (2)	25	24 (96)*	22 (88)*
Poor (3)	15	11 (73)*	8 (53)†
Totals	52	46 (88)	38 (73)

*,† Values with different superscripts are statistically different ($P < 0.05$; χ^2 analysis).

> 0.05; χ^2 analysis). However, fewer grade 3 embryos developed *in vitro* to hatching blastocysts when compared to grade 2 embryos (53 versus 88%, $P < 0.05$; χ^2 analysis). The difference in *in vitro* survival between grade 1 and grade 3 embryos, however, was not statistically significant (67 versus 53%).

An additional 17 embryos were vitrified in solution VS3a (method 3) and transferred into recipients immediately after thawing and in straw dilution (Table 2). All straws with the except of one contained a single embryo. The exception contained two embryos (one grade 2 and one grade 3). Seven (44%) of the recipients established a total of eight Day 90 pregnancies yielding an overall pregnancy rate of 47%. Although the pregnancy results suggest that embryo grade plays an important role in the success of *in vivo* development, none of the differences in pregnancy rate were statistically significant ($P > 0.25$; χ^2 analysis).

Experiment 2. The *in vitro* development of a total of 235 grade 1–3 morulae and early blastocysts was evaluated in a comparative evaluation of four cryopreservation methods (Table 3). Cryopreservation by methods 3 and 4 resulted in post-thaw survival rates comparable to that of method 1 ($P > 0.4$ and $P > 0.75$, respectively). However, the post-thaw survival rate of embryo cryopreserved according to method 2 was significantly lower than those of method 1 ($P < 0.10$) and method 4 ($P < 0.05$).

Further statistical tests by logistic ANOVA indicate that embryo grade, embryo stage, interval between embryo collection and cryopreservation, and cryopreservation method significantly affected the percentage of embryos that developed into expanded blastocysts ($P < 0.05$). Therefore, in statistical tests of significance of cryopreservation methods, the post-thaw survival rates *in vitro* for the cryopreservation methods were adjusted for three factors: (i) early blastocysts showed higher survival rates than morulae, (ii) percentage developing to expanded blastocysts was highest for grade 1 embryos and lowest for grade 3 embryos, and (iii) survival rates decreased when time interval between embryo collection and cryopreservation increased. Specifically, embryos cryopreserved 6–8 h after embryo collection showed significantly lower survival rates than embryos cryopreserved 0–6 h after embryo collection.

An additional 95 morulae and early blastocyst stage bovine embryos were cryopreserved by methods 1, 3, or 4 to assess their ability to develop *in vivo* (Table 4). Logistical ANOVA indicates that cryopreservation method is the only factor affecting pregnancy rates significantly in this study ($P < 0.05$), whereas embryo grade, devel-

TABLE 2
Pregnancies from Bovine Embryos after Vitrification in VS3a (Method 3), Rapid Warming, and One-Step Transfer (Experiment 1)

Embryo grade	No. of			
	Embryos vitrified	Recipients	Pregnant recipients (%)	Day 90 fetuses (%)
Excellent (1)	4	4	3 (75)	3 (75)*
Good (2)	6	6[a]	3 (50)	3 (50)*
Poor (3)	7	6	1 (17)	2 (29)*
Totals	17	16	7 (44)	8 (47)

[a] One recipient also received one grade 3 embryo; both embryos yielded a Day 100 fetus.
* Values do not differ significantly ($P > 0.25$; χ^2 analysis).

TABLE 3
In Vitro Development of Day 7 Bovine Embryos Following Cryopreservation by Four Methods (Experiment 2)

Preservation method[a]	Embryo grade	No. of Embryos	
		Cryopreserved	Developing to expanded blastocysts (%)
Method 1 (IETS Manual)	Excellent (1)	5	4 (80)
	Good (2)	50	25 (50)
	Poor (3)	12	3 (25)
	Totals	67	32 (48)*
Method 2 (Leibo, 1982)	Excellent (1)	8	4 (50)
	Good (2)	53	16 (30)
	Poor (3)	7	0 (0)
	Totals	68	20 (29)†
Method 3 (Rall, 1992)	Excellent (1)	12	8 (67)
	Good (2)	47	18 (38)
	Poor (3)	4	1 (25)
	Totals	63	27 (43)*,†
Method 4 (Massip, 1987)	Excellent (1)	2	2 (100)
	Good (2)	31	15 (48)
	Poor (3)	4	1 (25)
	Totals	37	18 (49)*

[a] See text for details of methods.
*,† Values with different superscripts are statistically different ($P < 0.10$; logistic ANOVA).

opmental stage of the embryo, quality of the recipient, and interval between embryo collection and cryopreservation did not ($P > 0.05$). Pregnancy rates after transfer of embryos cryopreserved according to method 1 (59%, $n = 34$) were higher than those for method 3 (43%, $n = 40$) and method 4 (24%, $n = 21$). However, the only statistically significant difference was found between methods 1 and 4 ($P < 0.025$).

TABLE 4
In Vivo Development of Day 7 Bovine Embryos Following Cryopreservation by Methods 1, 3, and 4 (Experiment 2)

Preservation method[a]	Embryo grade	No. of Embryos	
		Cryopreserved and transferred	Establishing Day 60 pregnancies (%)
Method 1 (IETS Manual)	Excellent (1)	7	3 (43)
	Good (2)	27	17 (63)
	Totals	34	20 (59)*
Method 3 (Rall, 1992)	Excellent (1)	4	2 (50)
	Good (2)	36	15 (42)
	Totals	40	17 (43)*,†
Method 4 (Massip, 1987)	Excellent (1)	2	0 (0)
	Good (2)	19	5 (26)
	Totals	21	5 (24)†

[a] See text for details of methods.
*,† Values with different superscripts are statistically different ($P < 0.05$; logistic ANOVA).

DISCUSSION

Our results indicate that vitrification in solution VS3a and one-step dilution (method 3) of Day 7 bovine embryos yields *in vitro* development and pregnancy rates that do not differ from those obtained using controlled slow freezing and stepwise dilution (method 1, $P > 0.10$). This result contrasts with that obtained when embryos were cryopreserved by standard slow freezing and diluted using a modification of Leibo's (10) one-step procedure (method 2) or vitrified by a modification of Massip *et al.'s* procedure (method 4). Embryos cryopreserved by method 2 yielded significantly lower rates of development *in vitro* and on this basis were excluded from the subsequent comparative embryo transfer study. The modifications of the one-step procedure by Leibo (10) involved the loading of the straw and the warming procedure. In our study the order of loading the cryoprotectant and sucrose diluent solutions into the straw was reversed from that of Leibo (10). The sucrose diluent was aspirated first and then followed by the cryoprotectant. One consequence of this change is the need to shake the straw twice during in-straw dilution, rather than once as per Leibo (10). Our warming rate of 1500°C/min (21) was higher than the 175–275°C/min used by Leibo (10). However, it is not likely that either modification had a negative effect on post-thaw survival.

Embryos cryopreserved by method 4 yielded a *in vitro* development rate that did not differ from those of methods 1 or 3. However, the rate of development *in vivo* was significantly lower than that of method 1. This result indicates that a high rate of development *in vitro* following bovine embryo cryopreservation is no guarantee for a high rate of development *in vivo*.

Several differences between the two vitrification methods may explain the higher efficacy of method 3. First, the type and total concentration of solutes in the vitrification solutions is different. The composition and concentration has an important effect on the cooling and warming rate requirements for successful vitrification (3). Solution VS3a (method 3) was formulated to yield a stable glassy state using moderate cooling and warming rates (respectively, $\geq$20 and $\geq$100°C/min; 17, 22), whereas Massip's vitrification solution (method 4) requires rapid cooling and warming (respectively, >100 and >500°C/min; 11, W. F. Rall, unpublished observations). This difference presumably reflects the higher macromolecule concentration in VS3a when compared to Massip's vitrification solution (respectively, 6 and 0.15%, w/v, BSA). A second difference is the concentration of Dulbecco's phosphate-buffered salts in the vitrification solution. Massip's vitrification solution was prepared by adding neat cryoprotectant to an isotonic saline. This approach maintains a constant concentration of salts by one measure, a molal basis (mol salt/1000 g H_2O), but reduces the saline component's concentration by about 50% on a molar basis (mol salt/litre solution). Our VS3a was formulated to maintain isotonicity on a molar basis. The molar method is preferable when the cryoprotectant concentration is greater than 4 *M*, as suggested by Meryman and Douglas (13). Recently, Papis *et al.* (15) compared both methods for maintaining the isotonicity of saline components for a rabbit embryo vitrification. They report that the "molar" method yields higher embryo viability postvitrification than the "molal" method.

Modifications of the original procedure of Massip *et al.* (11, 12) may account for our low rate of development *in vivo* (24%) compared to that reported by Massip and colleagues (39–54%). Our modifications were (i) the position of sucrose and vitrification solutions in the straw, (ii) the method used to seal the straw, (iii) the temperature of equilibration in the vitrification solution, (iv) the method used to cool embryos to the storage temperature, and (v) the warming

procedure. The first modification was to reduce the likelihood of contamination and dilution of the vitrification solution with the sucrose diluent (6). We aspirated all of the sucrose diluent into the straw first and then aspirated two smaller columns (0.5 cm) of vitrification solution separated by 0.5 cm air bubbles. Our aim was to use the first column of vitrification solution to remove any sucrose solution that may remain on the wall of the straw and reduce contamination of the second drop containing the embryo. This minimized the likelihood of ice crystal formation in diluted vitrification solution during cooling to the storage temperature as reported by Kobayashi *et al.* (6).

The second modification was heat sealing of both ends of the straw. We sealed straws rather than leaving them unsealed for two reasons: first, it ensured compliance with international regulations to prevent contamination of the embryo suspension during storage in liquid nitrogen (26) and shipping (28). Second, hermetic seals permitted the use of the one-step dilution of embryos without recovering embryos from the straw.

The third modification was to reduce the temperature of the vitrification solution in the straw to 4°C when the embryo was loaded into the straw. The equilibration temperature was reduced from room temperature to reduce the potentially harmful effects of chemical toxicity (W. F. Rall and M. J. Wood, unpublished observations) and/or further permeation of cryoprotectants into the embryos during heat sealing.

The fourth modification was to place the straw into cold nitrogen vapor (−170°C) for 2 or 3 min prior to transfer into liquid nitrogen. The cooling procedure was altered from direct transfer into liquid nitrogen for two reasons: first, cooling in cold nitrogen vapor reduces straw breakage due to the rapid crystallization of sucrose when straws are plunged directly into liquid nitrogen. Second, cooling in nitrogen vapor reduced the likelihood of zona and embryo damage due to fracturing of the glass during rapid temperature changes (21).

The final modification was the warming procedure. In Massip's original procedure, straws were placed directly into a water bath of 20°C, whereas in this study, straws were first held in room temperature air for 10 s, followed by 10 s in a 20°C water bath. Thawing in air first reduced the likelihood of zona and embryo damage due to fracturing of the glass during rapid temperature changes (21).

Although the pregnancy rate following vitrification in VS3a and one-step dilution (method 3) shows a trend to be slightly lower than that observed using conventional freezing and stepwise dilution (method 1), vitrification and one-step dilution offer several advantages for routine bovine embryo cryopreservation. First, no special controlled-rate freezing equipment is required for vitrification. Only a standard dissecting microscope, the vitrification solutions, and liquid nitrogen are required. This makes vitrification especially appropriate for use "on-the-farm." Although time required for equilibration and cooling is reduced using vitrification compared to conventional freezing, time is only saved in those cases when low numbers of embryos are cryopreserved (less than about 10). Second, the time required for removal of the glycerol is reduced to about 7 min which allows considerably more embryos to be transferred per time unit than with the current multistep dilution procedure. Third, the services of an embryologist are not required to recover and handle the embryos for thawing. Fourth, the equipment required to thaw and transfer one-step diluted embryos is limited. Only two water baths (36 and 22°C), a clock timer, and standard embryo transfer supplies are required, permitting transfer in remote locations. The inability of the embryo transfer practitioner to examine the embryo before transfer may

seem to be a disadvantage. In practice, approximately 1–5% of the embryos frozen according to the controlled slow freezing method are classified as nontransferable and discarded. In this study, all thawed embryos were cultured or transferred. However, a final advantage of the one-step procedure is that by not discarding grade 4 embryos after thawing, more calves will be produced per donor collection (16). The economic consequence of a slightly decreased pregnancy rate will, for the above reasons, mostly be compensated for by the speed and convenience of the vitrification and one-step dilution procedures. Other one-step procedures that have been reported to result in promising pregnancy rates in the cases of cattle and sheep embryos are the use of ethylene glycol or 1,2-propanediol as cryoprotectants (5, 29, 32). In the case of ethylene glycol, embryos were transferred directly upon thawing [no dilution (32)] or were diluted in the same straw in which they were cryopreserved using a sucrose solution prior to transfer (5). In the case of 1,2-propanediol, embryos were cryopreserved in the presence of 0.2 *M* sucrose and transferred directly upon thawing (29).

In conclusion, a comparison of the efficacy of four cryopreservation procedures for Day 7 bovine embryos indicates that vitrification using solution VS3a and subsequent dilution using a one-step procedure yields pregnancy rates similar to those of standard slow freezing and stepwise dilution.

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