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Status of Cryopreservation of Embryos from Domestic Animals[1]

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The discovery of glycerol as an effective cryoprotectant for spermatozoa led to research on cryopreservation of embryos. The first successful offspring from frozen–thawed embryos were reported in the mouse and later in other laboratory animals. Subsequently, these techniques were applied to domestic animals. Research in cryopreservation techniques have included studies concerning the type and concentration of cryoprotectant, cooling and freezing rates, seeding and plunging temperatures, thawing temperatures and rates, and methods of cryoprotectant removal. To date, successful results based on pregnancy rates have been obtained with cryopreserved cow, sheep, goat, and horse embryos but no success has been reported in swine. Post-thaw embryo survival has been shown to be dependent on the initial embryo quality, developmental stage, and species. The freezing techniques most frequently used in research and by commercial companies are identified as "equilibrium" cryopreservation. In this technique the embryos are placed in a concentrated glycerol solution (1.4 *M* in PBS supplemented with BSA) at room temperature and the glycerol is allowed to equilibrate for a 20-min period. During the cooling process the straws are seeded (−4 to −7°C) and cooling is continued at a rate of 0.3 to 0.5°C/min to −30°C when bovine embryos may be plunged into LN_2. Sheep embryos are successfully frozen with ethylene glycol (1.5 *M*) or DMSO (1.5 *M*) rather than with glycerol. Horse embryos have been frozen in 0.5 rather than 0.25 cc straws but with cooling rates and seeding and plunging temperatures similar to those used with bovine embryos. Swine embryos have shown a high sensitivity to temperature and cryoprotectants probably due to their high lipid content and a temperature decrease to 15 or 10°C causes a dramatic increase in the percentage of degenerated embryos. However, a recent study has shown that hatched pig blastocysts survived exposure below 15°C. Recent research has shown that embryos may also be frozen by a "nonequilibrium" method. This rapid freezing by vitrification consists of dehydration of the embryo at room temperature by a very highly concentrated vitrification media (3.5 to 4.0 *M*) and a very rapid freeze that avoids the formation of ice allowing the solution to change from a liquid to a glassy state. Vitrification solutions consist of combinations of sucrose, glycerol, and propylene glycol. With this technique, 50% pregnancy rates have been reported with the bovine blastocyst. © 1992 Academic Press, Inc.

The concept of embryo transfer was first reported in rabbits a century ago by Heape (36) and it was not until 1952 (136) and 1960 (2) that successful results were reported in the bovine. Embryo transfer techniques have been developed to provide live young in other species of domestic animals as well. In 1949, Polge (87) discovered glycerol as the cryoprotectant solution that allowed sperm survival after thawing and only 25 years later, successful reports on cryopreservation of bovine embryos were published. To date, the successful cryopreservation of the embryo has allowed: (i) genetic material to be moved around the world (29, 101–103, 116) and (ii) indigenous and exotic breeds to be preserved by developing embryo banks. Embryo freezing is essential for embryo transfer practitioners (30). The improvement in pregnancy rates obtained with thawed embryos now allows their long-term storage when their number

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exceeds the number of recipients available or when the live young need to be obtained at a later date or at another location. Successful cryopreservation of embryos in other domestic species has also been reported with live young obtained in sheep, goats, horses, and rabbits. Virtually no success has been achieved in cooling or freezing swine embryos. Embryos from a total of 15 mammalian species have been successfully cryopreserved. Emphasis on cryopreservation has focused on morula and blastocysts collected on Day 6 or 7 in cattle, sheep, and horses (132) or Day 6 in pigs (Day θ = day of standing heat).

Lately, interest in cryopreservation of oocytes and early embryonic stages is growing and it has been found that there is an increased tolerance to low temperature as compaction and blastulation occur (83, 135). Less advanced cow embryos fail to survive cooling to +15°C or below with the conventional freezing methods (118); however, instead, hatched blastocysts have survived freezing (76). This stage sensitivity has not been noted in one-cell to hatched sheep blastocysts; however, most attention has been directed to cryopreserving the later stages (132). The stage of development appears to be a critical factor for survival of equine embryos (111), with Day 6 embryos surviving better than Day 7 or 8 embryos (111, 141). Other factors have been mentioned as critical for the success of the embryo cryopreservation (77) including the initial embryo quality (43, 62, 80) and the time from embryo collection to the onset of freezing (103). There are also reports showing that good pregnancy rates may be achieved with bovine embryos cultured for 30 h before freezing (35).

Generally, embryo cryopreservation techniques used during the last decade include the following steps:

(i) Addition of cryoprotectant

(ii) Cooling of the embryo, induction of ice formation, freezing and storage in liquid nitrogen

(iii) Thawing of the embryo

(iv) Removal of cryoprotectant

The above methods allow permeation of cryoprotectant at room temperature and the dehydration of the embryos during the cooling and freezing processes before being plunged into liquid nitrogen. More recently, new approaches to long-term embryo preservation by dehydration at room temperature and by vitrification have been proposed. These two different approaches to cryopreservation of embryos have been defined as equilibrium and nonequilibrium cryopreservation. The objective of this paper is to review the pertinent literature on cryopreserving embryos of domestic animals and evaluate changes in methods that have improved pregnancy rates using thawed embryos.

EQUILIBRIUM CRYOPRESERVATION

Cryoprotectants and Their Method of Addition

The cryoprotectants used for embryo freezing usually penetrate cells. The most commonly used are glycerol, ethylene glycol, and dimethyl sulfoxide (DMSO). DMSO and ethylene glycol are more freely permeable than glycerol, and this is influenced by differences in the permeability characteristics of embryos from various species and at various stages of development (127).

During the initial prefreeze phase, the embryos are exposed and equilibrated to the cryoprotectant. Embryos exposed to a permeating cryoprotectant shrink by losing water until equilibrium is reached. This shrinkage is due to the initial hyperosmoticity of the extracellular solution and the fact that embryos are much more permeable to water than to cryoprotectants; shrinkage stops when an equilibrium is reached between the efflux of water and the influx of cryoprotectant (67, 68, 99, 100). As the additive permeates the embryo, it gradually reexpands because of the reentry

of water to maintain the osmotic equilibrium. The rate at which this reexpansion occurs reflects (i) the species of the embryo, (ii) the stage of embryonic development, (iii) the embryos' surface-to-volume ratio, (iv) the cryoprotectant itself, and (v) the temperature of exposure (55, 127).

The type of cryoprotectant agent used and its molarity differ from one species to another. DMSO at molarities of 1.0 to 1.5 has given successful results for freezing embryos (128, 133) while glycerol from 1.0 to 4.0 *M* has been used successfully (61). The two most commonly used cryoprotectants for mammalian cells are glycerol and DMSO; however, studies have shown no difference in survival of cattle embryos when 1.5 *M* ethylene glycol, 0.625 *M* dimethyl sulfoxide (DMSO) + 0.625 *M* glycerol, or 1.33 *M* glycerol were used (28) or between 1.4 *M* glycerol and 1.5 *M* DMSO added in three or one step (33, 34). The cryoprotective effects of glycerol, DMSO, and a mixture of both were compared in bovine embryos, and it was concluded that there was no difference in embryo survival between the use of the two cryoprotectants (9, 51, 52). However, another study showed that their combination (1.0 *M* glycerol + 0.5 *M* DMSO) was not as effective (53). Bouyssou and Chupin (13) observed higher survival rates of bovine embryos when frozen in glycerol than in DMSO. Table 1 illustrates the types of cryoprotectants, their concentration, and the method of addition that have been used in the different species of domestic animals.

The method of addition of the cryoprotectant also has been studied. Initially, the cryoprotectant was added in four or six steps (13, 43) involving 40–50 min for glycerolization before cooling and freezing. Later, the procedure could be reduced to three steps (94) without reducing embryo survival. Massip *et al.* (66) obtained a 45.2% pregnancy rate when embryos were frozen in 1.5 *M* DMSO added in three increasing concentrations steps and removed in six steps. Later, similar survival rates were reported with conventional stepwise and one-step addition to 7-day bovine embryos (23, 80).

A glycol, 1,2-propanediol, appears to be efficient in cryopreservation, is highly stable in the amorphous state, and thus limits ice formation. It also appears to be less toxic than DMSO and has been used in cryopreserving of early stage embryos. It has been suggested that the inclusion of 20% raffinose in combination with 1.4 *M* glycerol increases both *in vitro* survival and pregnancy rate of thawed bovine embryos (96). Also the addition of 0.1% Solcoseryl to the medium before freezing may beneficially affect post-thaw development of early bovine embryos (88).

Cooling of the Embryo, Seeding, and Freezing

In addition to the choice of cryoprotectant and its method of addition, the velocities at which embryos are cooled and rewarmed determine survival. The aim is to drive as much water as possible from the embryo and thus prevent intracellular ice formation while keeping the cytoplasm supercooled until freezing. Seeding induces a phase change from water to ice that brings an increase in the concentration of salts in the suspending solution. Once the ice forms, the embryos are cooled slowly, allowing them to respond osmotically to the concentration changes (56). The specific embryo cooling rate depends on the embryo surface to volume ratio, temperature and hydraulic conductivity, or the water permeability coefficient and the temperature dependence of that coefficient (55). The important role of cooling rate on cells during freezing was explained initially by Mazur (70) and has been reviewed by others (27, 31, 37, 55). Lehn-Jensen *et al.* (48) found that slow cooling produces a gradual shrinkage as intracellular water flows out of the cells and freezes extracellularly, reducing the embryo volume to 50% by −15°C

TABLE 1
Cryoprotectants and Their Methods of Addition to and Removal from Bovine Embryos

Cryoprotectant	Method of addition	Cryoprotectant concentrations	Method of removal	References	Year
DMSO	5 steps	0.25, 0.5, 1, 1.25, 1.5 *M*	6 steps	Trounson (117)	1978
DMSO	3 steps	N/A	6 steps	Lehn-Jensen (54)	1978
DMSO	3 steps	0.5, 1.0 and 1.5 *M*	6 steps	Massip (65)	1979
DMSO	3 steps	0.5, 1.0, 1.5 *M*	6 steps	Farrand (32)	1982
G	6 steps	0.2, 0.4, 0.6, 0.8, 1.0 *M*	1.0 *M* G + 0.5 *M* S, 0.5 *M* S	Nieman (80)	1982
G	3 steps	0.4, 0.9, 1.4 *M*		Bouyssou Chupin (13, 14)	1982
G, DMSO, EG	3 steps	1.5 *M* EG, 0.625 *M* DMSO + 0.625 *M* G, 1.33 *M* G		Elsden (28)	1982
G	Direct	1.4 *M*		Lehn-Jensen (46)	1983
G	1 step	1.5 *M* G	0.25 *M* S	Bousquet (12)	1983
G	1 step	1.5 *M*	1.08 *M* S	Leibo (58)	1984
G + S	1 step	1.5 *M* G + 1.0 *M* S	0.25 *M* S	Bui-Xang-Nguyen (15)	1984
DMSO and G	1 and 3 steps	1.5 *M* and 1.4 *M* resp	N/A	Franks (34)	1985
G + S	1 step	2.8 *M* G + 0.25 *M* S	1.0 *M* S	Chupin (21)	1986
G + PROH	2 steps	10% G + 20% PROH[a] and 25% G + 25% PROH	1.0 *M* S	Massip (63, 64)	1986
S + PROH	2 steps	1.5 *M* PROH for 30 min 1.0 *M* PROH + 0.1 *M* S 5 min and 0.05 *M* S 5 min or 2.0 *M* PROH + 0.5 *M* S	0.5 *M* S 10 min	Vincent (122)	1986
G + S	1 step	2.1 *M* G + 0.25 *M* S	1.0 *M* S	Chupin (20)	1987
G + PROH	2 steps	25% G + 25% PROH	1.0 *M* S	Bielanski (4)	1988
G + PROH	3 steps	3.4 *M* G + 3.4 *M* PROH	1.0 *M* S	Van Der Zwalmen (121)	1989

Note. G, glycerol; EG, ethylene glycol; S, sucrose; PROH, 1,2-propanediol; DMSO, dimethyl sulfoxide; *M*, molar concentration.
[a] Percentage cryoprotectant (v/v).

and to approximately 40% at −20°C. Conversely, if the cooling rate is too rapid, the cytoplasm eventually freezes. In early work slow freezing and thawing methods were used, based on the observation that the embryos have a low permeability to water and, therefore, must be cooled very slowly at rates below 1°C/min and rewarmed slowly (4–25°C/min). The embryos were usually plunged in liquid nitrogen at temperatures ranging from −60 to −120°C obtaining extensive dehydration. Later, faster methods were developed and embryos could be plunged in liquid nitrogen at temperatures between −30 and −40°C (50, 80, 107, 114, 115).

Initial reports indicated that bovine embryos may be frozen by a slow method. Lehn-Jensen (46) reported 43.2% pregnancies with embryos that were frozen in 1.5 *M* DMSO, cooled from room temperature to −7°C at 1°C/min at 0.3 to −36°C and then at 0.1°C/min to −60°C before plunging in liquid nitrogen. The same author also established that bovine blastocysts consistently survive plunging into liquid nitrogen after programmed freezing (0.3°C/min) to −20°C (46, 49), whereas survival was negligible when plunging occurred at −15°C, showing that dehydration of the embryo occurred from the seeding point down to approximately −20 to −25°C. Table 2 illustrates

the different cryoprotectants and freezing rates that have been used.

Takeda *et al.* (110) obtained no differences in embryo survival after embryos were plunged at temperatures between −25 and −35°C. However, embryos that were cooled from room temperature to seeding at 1°C/min had higher survival rates when plunged at −35°C than those plunged at other temperatures. Likewise, Farrand *et al.* (32) reported that more embryos survived when cooled at 0.3°C/min to −33, −38, or −43°C and plunged than those plunged at −19 or −50°C. Conversely, Zhenyan *et al.* (143) indicated no difference in survival rates when embryos were frozen by the slow or rapid freeze–thaw method using 1.5 *M* DMSO. Pettit (84) found no difference in pregnancy rate when bovine embryos were cooled at 0.3°C/min to −35°C and then at 0.1°C/min to −38°C or cooled from seeding to −38°C at 0.3°C/min and then plunged into LN_2. Bouyssou and Chupin (13) demonstrated that embryos cooled to −7°C, seeded, and later transferred to −30°C for 30 min before plunging in liquid nitrogen gave survival rates comparable to those obtained by the controlled freezing method. Others (82) reported pregnancy rates of at least 40% with embryos suspended in a solution of PBS with 1.2 *M* glycerol and 20% fetal serum cooled at a constantly reducing rate (from 10 to 0°C/min) for 60 min, exposed for 30 min at −30°C, and subsequently transferred to liquid nitrogen. Today, embryos are frozen commercially in straws (66, 12) or ampules. Bezugly *et al.* (3) demonstrated that bovine morula and blastocysts are not affected by temperature shock and may be cooled at temperatures of ~1000°C/min from 37 to 0°C without losing their viability *in vitro*. Ultrastructural findings showed that no damage was induced in blastomeres with rapid cooling (0.3–0.5°C/min to plunge temperatures of −30 to −40°C) and rapid thawing (~300–360°C/min) (41, 45).

Thawing of the Embryo

In general, embryos that are frozen by a slow method (slow cooling to temperatures of about −60°C) need to be thawed slowly (about 20°C/min); and embryos that are plunged in liquid nitrogen at warmer temperatures (−30 to −40°C) require rapid thawing (about 300°C/min) to obtain maximum survival. Embryos plunged from temperatures as high as −30 to −40°C still contain residual water that vitrifies and recrystallizes during slow warming causing severe intracellular damage (86). Recrystallization is avoided by rapid thawing. By contrast, embryos cooled slowly to −60°C are more dehydrated and recrystallization does not occur during slow warming (127).

Thawing embryos in air or their exposure to air for 6 s followed by immersion in water produces minimal damage to zona pellucidae (109). Cacheiro *et al.* (16) reported that bovine embryos frozen in 1.5 *M* DMSO and cooled at 0.5°C/min to −30°C and at 1.0°C to −68°C had higher survival rates when thawed at 20°C (81%) than at 37°C (50%). Likewise, Bilton (8) indicated that embryos cooled at 0.3°C/min to temperatures between −30 and −36°C and transferred directly to liquid nitrogen survived only when thawed rapidly (360°C/min), yet when embryos were transferred to liquid nitrogen at −42°C and below, they survived both slow and rapid thawing.

The incidence of zona damage to bovine ova frozen in straws is dependent on the warming conditions, with no damage observed when thawing is conducted at 20°C air (90); however, thawing in water baths at 20 or 36°C produced zona damage in 17 and 24% of the embryos, respectively.

Removal of Cryoprotectant

Direct transfers of nonfrozen embryos in glycerolated PBS (50) as well as of frozen

TABLE 2
Effect of Cooling and Freezing Rates on Survival and Fertility of Bovine Embryos

Room temperature to seeding temperature	Holding time and seeding temperatures	Seeding to plunge	Final cryoprotectant concentration	Percentage pregnancy	Reference	Year
Direct	−7°C, 5 min	0.3°C to −80°C	1.5 *M* DMSO	4/23	Trounson (117)	1978
1°C/min	−6°C	0.3°C/min to −36°C, 0.1°C to −60°C	1.5 *M* DMSO	43.8	Lehn-Jensen (52, 53, 54)	1978, 1979, 1980
1°C/min	−6°C	0.3°C/min to −30°C, 0.1 to −33°C	1.5 *M* DMSO	45.2	Massip (65)	1979
1°C/min	−6°C	0.3°C/min to −20°C	1.4 *M* G	56	Lehn-Jensen (51)	1981
1°C/min	−7°C	0.3°C/min to −30, 0.1 to −33°C	1.5 *M* DMSO	N/A	Tervit (114)	1981
1°C/min	−7°C	0.3°C/min to −30, 0.1 to −33°C	1.5 *M* DMSO	6/20	Tervit (115)	1981
1°C/min	−4°C	0.3°C/min to −40°C	1.0 *M* G	N/A	Smorag (108)	1981
1°C/min	−7°C	0.3°C/min to −30 to −50°C	1.5 *M* DMSO	N/A	Farrand (32)	1982
1°C/min	−7°C	0.3°C/min to −28, 0.1 to −35°C	1.0 *M* G	N/A	Nieman (80)	1982
Direct	−7°C, 8 min	1.3°C/min to −30°C or	1.4 *M* G	N/A	Bouyssou (14), Chupin (21)	1982
Direct	−7°C, 8 min	Directly to −30°C for 30 min	1.4 *M* G	N/A	Chupin (21)	1982
Direct	−5°C, 10 min	0.3°C/min to −30°C	1.4 *M* G	57	Lehn-Jensen (46)	1983
1°C/min	−4°C	0.3°C/min to −35°C	1.0 *M* G	53	Utsumi (120)	1983
5°C/min	−7°C	0.3°C/min to −35°C	1.5 *M* G	N/A	Bousquet (12)	1983
1°C/min	−4°C	0.3°C/min to −40°C	1.0 *M* G	N/A	Del Campo (26)	1983
1°C/min	−4°C	0.3°C/min to −35°C	1.0 *M* G	53	Yuding (142)	1984
12°C/min	—	12°C/min to −30°C	1.5 *M* G + 1.0 *M* S	33.3	Bui-Xuan-Nguyen (15)	1984
1°C/min	−4°C	0.3°C/min to −40°C	1.0 *M* G	N/A	Bievich (6)	1985
4°C/min	−5°C, 15 min	0.5°C/min to −30°C or 1°C/min to −25, −30, −35°C	10% G[a]	54	Takeda (110)	1985
1°C/min	−6°C	0.3°C/min to −35, 0.1°C to −38°C or 0.3°C/min to −38°C	10% G	54	Pettit (84)	1985
1°C/min	−7°C	0.3°C/min to −30, 0.1 to −33°C	10% G	74.4	Christie (19)	1986
N/A	−7°C	0.3°C/min to −35°C	1.5 *M* G	51.8	Heyman (39)	1986
1°C/min	−7°C	0.3°C/min to −28°C, 0.1 to −35°C	1.4 *M* G	51	Nieman (78)	1986
—	Neck LN_2 tank, 5 min	Direct plunge into LN_2	2.8 *M* G + 0.25 *M* S	N/A	Chupin (21)	1986
0.5°C/min	−6°C	0.5°C to −30°C, 0.1 to −68°C	1.5 *M* DMSO	N/A	Cacheiro (16)	1986
—	—	Direct plunge into LN_2	10% G + 20% PROH, 25% G + 25% PROH	53.8	Massip (63, 64)	1986
—	—	Placed at −25°C for 90 min	2.0 *M* PROH + 0.5 *M* S or 1.0 *M* PROH + 0.1 *M* S		Vincent (122)	1986
Direct	−5°C	0.5°C/min to −30°C	1.4 *M* G	62.2	West (126)	1987
—	Neck LN_2 tank, 5 min	Direct plunge into LN_2	2.1 *M* G + 0.25 *M* S	N/A	Chupin (20)	1987
10 to 0°C at constant rate for 60 min	Spontaneous not < −6°C	Directly placed at −30°C Held for 30 min	1.2 *M* G	Not <40%	Ostahko (82)	1988
—	—	Direct plunge into LN_2	25% G + 25% PROH	N/A	Bielanski (4)	1988
—	—	Direct plunge into LN_2	3.4 *M* G 3.4 *M* G + 0.25 *M* S 3.4 *M* G + 3.4 *M* PROH		VanDerZwalmen (121)	1989

Note. G, glycerol; EG, ethylene glycol; S, sucrose; PROH, 1,2-propanediol; DMSO, dimethyl sulfoxide; M, molar concentration.
[a] Percentage of cryoprotectant (v/v).

embryos without glycerol removal have yielded poor results (133). Early reports indicate that the cryoprotectant may be removed after thawing in 0.25 *M* steps by transferring the embryos through PBS solutions of decreasing glycerol concentrations until they are in PBS with serum. In 1982, Renard *et al.* (95) and very shortly after Leibo (60) introduced a new method for removing glycerol by including a 0.25 *M* sucrose solution in the straw (Table 1). They postulated that the glycerol would diffuse passively to the exterior of the cells and sucrose, which could not permeate the cells, would prevent the osmotic shock during the dilution steps. Leibo (60) reported that frozen–thawed bovine embryos may be successfully diluted in one-step within the straw where they were previously frozen for direct nonsurgical transfer yielding 36.7% pregnancy rate at 90 days (58, 59) and 45.2% in a later field trial (57). In an independent study, Chupin *et al.* (22, 23) found that there was no statistical difference between the two methods. Takeda *et al.* (110) and Prather *et al.* (89) compared the removal of glycerol by sucrose solution in three steps or by the six-step method commonly used with sucrose and found no significant differences between the two protocols. Conversely, Seok *et al.* (101) obtained a higher conception rate when glycerol was removed in six steps than with a two-step sucrose gradient (36.7 and 16.7%, respectively).

In vitro survival of bovine embryos at 4 h was significantly higher after glycerol removal with one-step sucrose when compared with the stepwise method (5, 80). And Renard *et al.* (93) evaluated the efficiency of the technique by concluding that the ratio of frozen embryos to fresh embryos that survived was the same 70.9/87.5 *in vitro* and 46.8/57.7 *in vivo*.

Tervit and Goold (113) compared survival of sheep embryos when the cryoprotectant was removed stepwise or by the sucrose method and observed that the latter was beneficial. No difference was obtained when the sucrose method was used in one or two steps (71). Later, Tervit (112) observed that a two-step sucrose gradient increased embryo survival over a three-step method obtaining 54% vs 38.5% pregnancy rates. Merry *et al.* (72) reported that stepwise cryoprotectant dilution procedures are superior to dilution with 0.25 *M* sucrose. And Ware and Boland (125) observed that a 1.0 *M* sucrose solution was optimum for embryos frozen in 1.4 or 2.0 *M* glycerol for the removal of cryoprotectant in one step.

NONEQUILIBRIUM CRYOPRESERVATION

Rapid Freezing

In 1981, Kasai *et al.* (42) found an increase in the survival of mouse embryos when stored at 0°C in solutions containing sucrose. Dehydration of the embryos also may be achieved before cooling by using an external cryoprotectant, such as sucrose, which creates a hyperosmotic environment. A new and simple method for freezing bovine embryos was developed by predehydrating them at room temperature in a solution containing 1.5 *M* glycerol + 1.0 *M* sucrose (15). The embryos are placed in the precooled straws (−30°C) already filled with freezing medium. The embryos are held at −30°C for 30 min before being plunged into liquid nitrogen (cooling rate = 12°C/min). This procedure does not require seeding of extracellular medium or computerized cooling rates. This method yielded a 33.3% pregnancy rate as compared with a 48.5% pregnancy rate obtained with embryos frozen by the conventional method. Later, Vincent and Heyman (122) equilibrated one- and two-cell bovine and rabbit embryos for 30 min in mixtures of 1,2-propanediol and sucrose (1.0 and 0.1 *M* or 2.0 and 0.5 *M*, respectively), before placing the embryos for 90 min in precooled straws at −25°C. The straws then were plunged into liquid nitrogen. They observed that partial dehydration and exposure to −25°C

were not detrimental to cattle blastocysts; however, the step between −25 and −196°C appeared to be critical as only 34.6% thawed embryos cleaved further *in vitro*. One pregnancy was obtained.

Chupin (21) reported quick freezing of expanding bovine blastocysts by incubating them in a 1.4 *M* glycerol solution and subsequent dehydration in 2.8 *M* glycerol + 0.25 *M* sucrose in PBS at room temperature. The same author (20) showed that 67.7% Day 7 blastocysts survived quick freezing when dehydrated for 2 min at room temperature in a mixture of 2.1 *M* glycerol and 0.25 *M* sucrose. With this method, crystallization was obtained by holding the straw vertically in the neck of the liquid nitrogen container for 5 min before plunging it into liquid nitrogen.

Heyman *et al.* (40) demonstrated that one-cell rabbit eggs partially dehydrated by a 0.5 *M* sucrose solution survived 24-h storage in the supercooled state provided that the water content was reduced to about 40%. Likewise, Renard *et al.* (95) showed that two-cell bovine embryos can be successfully frozen by a rapid procedure derived from the two-step freezing method described earlier by Wood and Farrant (139) involving mouse embryos.

Vitrification

Rall and Fahy (91, 92) recently described the process of vitrification in which crystalline ice does not separate and consequently the solutes are not concentrated: there is an increase in viscosity, producing a solid, glassy state. Using highly concentrated solutions of cryoprotectants that become so viscous that they pass from the liquid state to a nonstructured solid state, glass, they obtained 87.7% survival of mouse embryos when frozen at ~3000°C/min. The vitrification solution contained DMSO, acetamide, propylene glycol, and polyethylene glycol. With this method, the cooling rate is relatively unimportant, but initial exposure to the vitrifying mixture has to be less than 15 min at 4°C to reduce toxicity. Warming, however, has to be rapid to prevent crystallization as the temperature returns to normal. Glass formation is thought to be a general feature of all liquids when sufficiently high cooling rates and small sample sizes are used (70). Later, Kono *et al.* (44) froze rat blastocysts by this method using a solution that consisted of DMSO, acetamide, propylene glycol, and polyethylene glycol (VS1) (Vitrification Solution VS1) obtaining 100% development of the embryos after thawing to expanded blastocysts. The authors also indicated that vitrification of goat and cattle blastocysts in VS1 was examined according to the original procedure (90, 91) but failed to obtain sufficient results. If the disadvantages of the high concentrations of cryoprotectant mixtures are overcome, toxicity (which appears to be temperature dependent) is controlled, and good embryo recovery after thaw is obtained, vitrification could prove to be practical from a commercial standpoint. This would reduce the cost of embryo freezing by simplifying processing and eliminating the need for costly computerized freezing units.

Massip *et al.* (63, 64) first reported a 53% pregnancy rate (7/13) at 60 days with bovine morula–early blastocysts frozen by a vitrification method; however, the more advanced blastocysts did not survive in culture or *in vivo*. The embryos were equilibrated for 10 min at room temperature in PBS containing 10% glycerol + 20% 1,2-propanediol and later placed in a small drop (1 cm) in a ¼-ml French straw in a vitrification solution composed of 25% glycerol + 25% 1,2-propanediol and slowly plunged in liquid nitrogen. Van Der Zwalmen *et al.* (121) described a procedure that allows survival of blastocysts frozen by vitrification. The embryos were first exposed at room temperature to a solution of 3.4 *M* glycerol in PBS (25%) for 13 min and then to a so-

lution of 3.4 *M* glycerol + 0.25 *M* sucrose in PBS for 7 min. The blastocysts were then transferred to a drop of 3.4 *M* glycerol + 3.4 *M* 1,2-propanediol in PBS precooled at 4°C inside the straw and within 30 s the straws were plunged into liquid nitrogen. With this method they reported 7 pregnancies of 14 transfers at 90 days. They indicated a stage-dependent sensitivity of the embryos to the composition and concentration of the vitrification solutions. Bielanski and Hare (4) froze bovine demi-embryos in a vitrification solution (25% glycerol + 25% 1,2-propanodiol) and observed that they survived equally as well as those frozen by conventional methods.

SHEEP AND GOAT EMBRYOS

In 1959 Averill and Rowson (1) were the first to report the freezing of sheep embryos to −79°C with no success. In 1974 Willadsen *et al.* (136) reported the birth of the first live offspring from frozen–thawed sheep embryos (Table 3).

The choice of the cryoprotectant is dependent on the embryonic stage and on the species. Moore and Bilton (73) studied the toxicity of different concentrations of cryoprotectants to sheep embryos and observed that glycerol may be used as an effective cryoprotectant (7). However, a 2.0-*M* solution inhibited further development in culture (Table 4). Nieman (78) compared the survival of sheep embryos frozen in 1.5 *M* DMSO and 1.5 *M* glycerol and reported considerably more embryos viable when frozen in glycerol but found a difference in developmental stages. Survival rates for morulae were only 50%, whereas 93.3% of blastocysts were considered viable. Likewise, embryos frozen in 1.5 *M* DMSO, ethylene glycol, or propylene glycol survived more readily than those frozen in 1.4 *M* glycerol (100, 116). Significant differences ($P < 0.01$) were obtained in the survival of sheep embryos when frozen in 1.5 *M* ethylene glycol or 1.4 *M* glycerol, obtaining 62.5 and 23.3% survival, respectively (24).

No difference in embryo survival was observed when the cryoprotectant (1.5 *M* ethylene glycol) was added in one or two steps (71). Bilton and Moore (10) demonstrated the importance of seeding and reported no differences in sheep embryo survival when they were seeded at −2.5, −5, or −7.5°C; however, no embryos survived when seeded at −10°C. Embryos may be cooled successfully at 1°C/min from room temperature to seeding temperature (−7°C), at 0.3°C/min to −35°C and then at 0.1°C/min to −38°C (116) or cooled at 0.3°C/min to −30°C before plunging in liquid nitrogen (71, 136). Pregnancy rates of 35–55.5% may be expected (130).

Bilton and Moore (11) were the first to report the birth of live offspring from fro-

TABLE 3
Cryoprotectants and Their Methods of Addition to and Removal from Sheep and Goat Frozen Embryos

Cryoprotectant	Method of addition	Cryoprotectant concentrations	Method of removal	References	Year
Sheep					
G	3 steps	0.47, 0.93, 1.4 *M*	6 steps	Merry (72)	1984
G	3 steps	1.4 or 2.0 *M*	1.0 *M* S	Ware (125)	1987
EG	3 steps	0.5, 1.0, 1.5 *M*	0.25 *M* S	Cocero (24)	1988
EG	1 or 2 steps	1.5 *M*	0.25 and 1.0 *M* S or 1.0 *M* S	McGinnis (71)	1989
Goats					
G	1 step	1.0 *M* G	4 steps	Rong (97)	1989

Note. G, glycerol; EG, ethylene glycol; S, sucrose; *M*, molar concentration.

TABLE 4
Effect of Cooling and Freezing Rates on Survival and Fertility of Sheep and Goat Embryos

Room temperature to seeding temperature	Holding time and seeding temperatures	Seeding to plunge	Final cryoprotectant concentration	Percentage pregnancy	Reference	Year
Sheep						
1°C/min	−6°C, 2 min	0.3°C/min to −35°C	1.4 *M* G	N/A	Bilton (7)	1983
1°C/min	−7°C	0.3°C/min to −35°C, 0.1 to −38°C	1.5 *M* DMSO	50	Tervit (113)	1984
			or 1.5 *M* EG	57	Tervit (113)	1984
			or 1.4 *M* G	35	Tervit (113)	1984
1°C/min	−7°C, 2 min	0.3°C/min to −60°C	1.4 *M* G	N/A	Merry (72)	1984
1°C/min	−7°C	0.3°C/min to −28°C, 0.1 to −35°C	1.4 *M* G	50	Nieman (78)	1986
N/A	−7°C	0.3°C/min to −35°C	1.5 *M* EG	53.8	Heyman (39)	1986
Direct	−7°C, 1 min	0.3°C/min to −30°C	1.4–2.0 *M* G	N/A	Ware (125)	1987
2°C/min	−7°C	0.3°C/min to −30°C	1.5 *M* EG	N/A	Cocero (24)	1988
1°C/min from 0	−6.5°C, 10 min	0.3°C/min to −34°C for 20 min	1.5 *M* EG	N/A	McGinnis (71)	1989
Goat						
0.3°C/min	−6°C, 90 sec	0.3°C/min to −35°C	1.4 *M* G	68	Chemineau (18)	1986
1.0°C/min	−7°C	0.3°C/min to −36°C	1.0 *M* G	50	Wang (124)	1988
1.0°C/min	−6.5°C	0.5°C/min to −30°C	1.0 *M* G	53	Rong (97)	1989

Note. G, Glycerol; EG, Ethylene glycol; DMSO, dimethyl sulfoxide; *M*, molar concentration.

zen–thawed goat embryos. Other workers demonstrated that goat embryos survive freezing in 1.0 *M* glycerol solution (18, 97, 124) and have been frozen with success in Menezo medium with 1.4 *M* glycerol, seeded at −6°C, and cooled at 0.3°C/min to −35°C and at 30°C to −150°C before being plunged in liquid nitrogen. Four pregnancies of 6 transfers were reported by Tsunoda *et al.* (119), and later, 7 kids were obtained after transfer of 14 frozen–thawed embryos frozen by a faster method in 1.0 *M* glycerol in PBS and cooled at 0.5°C/min to a plunge temperature of −30°C (97).

PIG EMBRYOS

Porcine embryos have been difficult to freeze and to date no success has been reported. Nieman (78) observed that porcine embryos tolerated osmotic changes due to addition of different cryoprotectants; however, they did not tolerate temperature changes to even 10°C. These data are consistent with results from Polge and Willadsen (85). This is probably due to their high lipid content (38), but when a lipid stabilizer like phosphatidylcholine is added, this sensitivity can not be overcome (79). Willadsen (132) indicated that expanding pig blastocysts acquire sufficient tolerance to low temperatures and are able to survive and hatch during culture at 0°C. He indicated that such cooled embryos lose most of the large perinuclear lipid globules normally present and then are not viable when transferred to recipients.

Nagashima *et al.* (74, 75) reported post-thaw survival of expanded pig blastocysts (48.5%) and of hatched blastocysts (47.3%) on the basis of their noticeable expansion of the blastocoele when the embryos were frozen in 1.5 *M* DMSO, cooled from room temperature to −5°C at 1°C/min, seeded, cooled at 0.3°C/min to −20°C, and then immersed in liquid nitrogen. They concluded that transition of the state of development or differentiation may be accompanied by a change in low temperature sensitivity of pig embryonic cells.

EQUINE EMBRYOS

The primary use of embryo transfer in horses is to obtain foals from valuable, older mares or from mares that are in training and in show competition. Equine Day 6 embryos have been frozen with success in 1.0–1.32 *M* glycerol solution (106, 111, 140, 141).

Yamamoto and co-workers (140, 141) reported the first successful birth of a foal from a frozen embryo after its storage for 2 days at −196°C. Later, equine embryos were frozen successfully in 10% glycerol in PBS, added in two steps. Embryos were cooled to seeding temperature (−6°C) at 4°C/min and then at 0.3 to −33°C. Pregnancy rate at 50 days postovulation was 53% (9/17) (105), and smaller Day 6 embryos withstood cryopreservation in straws better than larger Day 7 embryos (106, 117, 138). Wilson *et al.* (138) indicated that the relationship between age and size of the

TABLE 5
Methods of Addition and Removal of Cryoprotectants in Equine Preimplantation Embryos

Cryoprotectant	Method of addition	Cryoprotectant concentrations	Method of removal	References	Year
G	3 steps	1.32 *M*	6 steps	Takeda (111)	1984
G	4 steps	10%[a]	4 steps	Cztonkowska (25)	1985
G	2 steps	10%	6 steps	Slade (105)	1985
G	2 steps	10%	1.0 *M* S	Wilson (138)	1986

Note. G, glycerol; S, sucrose; *M*, molar concentration.
[a] Percentage cryoprotectant (v/v).

TABLE 6
Effect of Cooling and Freezing Rates on Survival and Fertility of Preimplantation Embryos from Horses and Swine

Room temperature to seeding temperature	Holding time and seeding temperatures	Seeding to plunge	Final cryoprotectant concentration	Percentage pregnancy	Reference	Year
Equine						
1°C/min	−7°C	0.3°C/min to −26, 0.1 to −38°C	1.32 *M* G	50	Takeda (111)	1984
4°C/min	−6°C	0.3°C/min to −30, 0.1 to −33°C	10% G	53	Slade (105, 106)	1985, 1984
1°C/min	−6°C	0 to 3°C/min to −37 or to −40°C	10% G	N/A	Cztonkowska (25)	1985
Direct	−6°C, 5 min	0.3°C/min to −30°C	10% G	20	Wilson (138)	1986
Swine						
1°C/min	−5°C	0.3°C/min to −20°C	1.5 *M* DMSO	N/A	Nagashima (74)	1989

Note. G, glycerol; DMSO, dimethyl sulfoxide; *M*, molar concentration.
[a] Percentage cryoprotectant (v/v).

embryo plays an important part in their post-thaw survival. Also, Takeda *et al.* (111) reported no difference in embryo survival when they used slow and fast freezing rates, and two fillies were born after transfer of four embryos into three recipients. Equine embryos have been frozen in 0.5-ml French straws, ampules, (105) or small glass test tubes (25) and the higher survival results were obtained when frozen in straws (138). However, attempts at freezing have been limited in part due to lack of acceptance from the large breed associations (123). The embryos are generally thawed in water baths at 37°C (25, 105, 111) or at 25°C (138) (Tables 5 and 6).

SUMMARY

In summary, successful results based on pregnancy rates have been obtained with cryopreserved cow, sheep, goat, and horse embryos but no success has been reported in swine. The process of cryopreservation involves several steps such as addition of cryoprotectant, cooling of the embryos, induction of ice formation, freezing and storage of the embryo, thawing of the embryo, and removal of the cryoprotectant. Every one of these steps has been discussed in the article with special reference to the bovine, equine, ovine, and caprine embryo.

The cryopreservation process includes the equilibration of the embryo in the solutions that contain the cryoprotectant (DMSO, ethylene glycol, or glycerol) and is referred to as equilibrium cryopreservation. The alternative process where the embryo is exposed to the cryoprotectant for a very short period of time and the initial dehydration is accomplished by osmotic changes (addition of sucrose) is referred to as nonequilibrium cryopreservation and includes the so-called methods of quick freezing and vitrification. Some pregnancies have been obtained with the latter methods.

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