ABSTRACTS FOR POSTER PRESENTATION

Cryopreservation/Cryobiology

81 IN VITRO MATURATION, FERTILIZATION AND DEVELOPMENT OF BOVINE IMMATURE OOCYTES CRYOPRESERVED BY VITRIFICATION WITH STEPWISE EXPOSURE USING A NYLON MESH

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Vitrification of bovine immature oocytes has been reported using an open pulled straw, but with limited success. In a previous report, we developed an alternative material (nylon mesh) for vitrification of large quantities of oocytes and embryos. This study was conducted to demonstrate effects of components of a cryoprotectant and a protocol of exposure for bovine immature oocytes on their subsequent in vitro maturation, fertilization and development after cryopreservation by vitrification using a nylon mesh. Bovine oocytes at the germinal vesicle stage were collected from 2–5 mm follicles in ovaries, and cumulus-oocytes complexes (COCs) were randomly assigned to treatment groups. Before vitrification, COCs were exposed to the cryoprotectant, which was composed of 40% ethylene glycol, 18% ficoll and 0.3 M sucrose (EFS40) or 0.3 M trehalose (EFT40) by single step or stepwise exposure. Forty COCs were transferred onto a nylon mesh (0.5 cm^2), which was then plunged directly into liquid nitrogen. After thawing in warm medium, vitrified COCs were in vitro-matured, fertilized and cultured. After culture for in vitro maturation, the rates in the oocytes reaching to metaphase II were 64.1% and 63.1% in the stepwise exposure to EFS40 or EFT40, respectively, which was significantly higher ($P < 0.05$) than the corresponding rates after a single step (22.6% and 10.0%, respectively). There was no significant effect of the two sugars on in vitro maturation after single or step-wise equilibration. Transmission electron microscopy revealed that the cytoplasm of oocytes equilibrated in a single step had many vacuoles and broken mitochondria, while oocytes equilibrated in a step-wise manner had significantly fewer abnormalities and were similar to untreated controls. Cleavage rate of thawed oocytes after IVMFC was significantly higher after stepwise exposure to EFS40 or EFT40 than that after single step exposure (37.7% and 22.2% v. 20.8% and 0%, respectively, $P < 0.05$). Step-wise equilibration of oocytes in EFT40 was dramatically detrimental: no cleaved embryos developed to blastocysts after a single step exposure to either vitrification solution, or stepwise exposure to EFT40. However, blastocysts were obtained following stepwise exposure to EFS40 (8%). These results suggest that stepwise equilibration and vitrification on a nylon mesh minimizes structural damage to the organelles of immature oocytes and facilitates successful cryopreservation.

82 COMPARISON OF THE SURVIVAL OF IN VITRO-DERIVED PORCINE OOCYTES AND EMBRYOS VITRIFIED BY OPEN PULLED STRAW, ELECTRON MICROSCOPE GRID, AND NYLON LOOP SYSTEM

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Various sample containers have been developed for ultra-rapid vitrification of oocytes and embryos to minimize chilling and other injuries. In this study, the survival rates of in vitro-derived porcine oocytes were compared after cryopreservation using open pulled straw (OPS), electron microscope

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grid (EMG), and nylon loop system (NLS). In addition, the post-thaw survival of porcine morulae and blastocysts was assessed after vitrification by the OPS method. In vitro maturation and fertilization were performed according to the procedures of Funahashi *et al.*, 1994 Theriogenology 41, 1425–1433). Fertilized oocytes were cultured in glucose-free NCSU 23 supplemented with 5 mM sodium pyruvate, 0.5 mM sodium lactate and 4 mg mL^{-1} bovine serum albumin for 2 days at 39°C; 10% fetal bovine serum was added to the culture medium thereafter. Oocytes and embryos were treated with 7.5 μg mL^{-1} cytochalasin B for 30 min, centrifuged at 13,000*g* for 13 min and then exposed sequentially to an ethylene glycol (EG) vitrification solution (0 M for 1 min, 2 M for 5 min and 8 M plus 7% polyvinylpyrrolidone for 1 min). Oocytes ($n = 100$ per treatment group) were placed in or on sample containers and plunged into liquid nitrogen. Porcine morulae and blastocysts ($n = 137$) were similarly treated, aspirated into OPSs, and plunged into liquid nitrogen. Oocytes and embryos were thawed rapidly by transferring the sample container into 1 M EG for 2 min, and then the embryos were serially diluted by transfer into 0.5 M for 2 min, and medium for 5 min. Post-thaw survival of vitrified oocytes was assessed by development after IVF and culture to morulae or blastocysts. Post-thaw survival of vitrified morulae and blastocysts was assessed by their ability to continue development to, respectively, blastocysts and expanded blastocysts. After oocytes were thawed, fertilized and cultured in vitro, the rates of development to the morula- or blastocyst-stage were 8%, 0% and 6% for, respectively, the OPS, EMG and NLS groups. The rate of development of non-cryopreserved control oocytes was 38% (38/100). Although significantly fewer oocytes developed after vitrification compared to the control ($P < 0.05$), OPS and NLS containers were superior to EMG containers ($P < 0.05$). Morula- or blastocyst-stage embryos vitrified using OPS yielded a significantly higher rate of survival than did oocytes (48%, 66/137; $P < 0.05$). This study indicates that very few in vitro-matured porcine oocytes survive vitrification using OPS, EMG and NLS methods. Considerably higher rates of survival were obtained by in vitro-produced porcine morulae and blastocysts following vitrification by the OPS method. Additional research is needed to identify and develop methods to overcome the factors limiting the cryopreservation of porcine oocytes for practical uses. Supported by KOSEF (R05-2002-000-00388-0) grant.

83 ANALYSIS OF RAPID COOLING V. SLOW COOLING COMBINED WITH ICE CRYSTAL SEEDING FOR CRYOPRESERVATION OF PRIMATE EMBRYONIC STEM CELLS

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Primate embryonic stem (ES) cells have the ability to self-renew indefinitely while maintaining the ability to differentiate. This unique property allows scientists to study the factors necessary for stem cell self-renewal and differentiation in vitro that reflect in vivo processes. Work with primate ES cells is handicapped by the poor survival (1–5%) of rhesus and human ES cells following standard tissue culture methods of rapid cryopreservation. The purpose of this study was to compare and contrast two cryopreservation techniques, slow cooling combined with ice crystal seeding commonly used for mammalian embryos v. rapid cooling commonly used for tissue culture, to find a method for efficient primate ES cell cryopreservation. A combination of trials was run to compare dimethyl sulfoxide (DMSO) v. ethylene glycol as a cryoprotectant, a cooling rate of 0.3°C per minute following ice crystal seeding at −7°C v. placement at −80°C with no seeding, and rapid thaw with step-wise cryoprotectant removal v. one-step sucrose cryoprotectant removal. Cell survival was assessed through a combination of cell surface markers, alkaline phosphatase staining and morphology to look for undifferentiated cells and quantitate survival. All cryopreservations were performed with the same cell density. The survival of the cells with slow embryo-style cooling in DMSO with a step-wise cryoprotectant removal was 64.0% v. 12.8% with rapid cooling.

84 METHANOL AS A CRYOPROTECTANT FOR EQUINE EMBRYOS

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Equine embryos with diameters >300 μm have low survival rates post-thaw, possibly due to low permeability of the cryoprotectant glycerol. Methanol has been used successfully for freezing ovine and murine embryos. The objectives of this study were: (1) examine the effect of methanol as a cryoprotectant for large equine embryos; (2) determine the diameter change of embryos exposed to methanol compared to glycerol; and (3) compare pregnancy rates of embryos cultured in vitro prior to transfer. Equine embryos ($n = 43$) were recovered nonsurgically 7 to 8 days after ovulation and randomly assigned to be cryopreserved in one of two cryoprotectants: 4.8% v/v methanol ($n = 22$) or 10% v/v glycerol ($n = 21$). Embryos (300 μm to 1000 μm) were measured at 5 time points after exposure to glycerol (0, 2, 5, 10, and 15 min) or methanol (0, 1.5, 3.5, 7.5 and 10 min) to determine changes in diameter over time (% ± SD). Embryos were loaded into 0.25-mL plastic straws, sealed, placed in a programmable cell freezer and cooled from room temperature (22°C) to −6°C. Straws were then seeded, held at −6°C for 10 min, and cooled at 0.3°C/min to −30°C and then at 0.1°C/min to −33°C before being plunged into liquid nitrogen. Sets of three straws within a treatment group were thawed in air for 10 s and then immersed in a 38°C water bath for 20 s. Each set of three embryos was further assigned to be either cultured for 12 h prior to transfer or transferred nonsurgically to a single mare immediately. Embryo diameter decreased in all embryos upon initial exposure to cryoprotectant. Embryos in methanol shrank and then recovered slightly to 76 ± 8% of their original diameter; however, embryos in glycerol continued to shrink, reaching 57 ± 6% of their original diameter prior to cryopreservation. Survival rates of embryos through Day 16 of pregnancy were 38% and 23%, respectively, ($P > 0.05$) for embryos cryopreserved in the presence of glycerol or methanol. There was no difference in pregnancy rates of mares receiving embryos that were cultured prior to transfer or not cultured ($P > 0.05$). Methanol provided no advantage over glycerol as a cryoprotectant for equine blastocysts. Neither cryoprotectant provided satisfactory pregnancy rates of frozen/thawed large equine embryos. Further studies are needed to develop procedures for freezing large equine embryos.

85 ASSISTED HATCHING IMPROVES POST-WARMING IN VITRO VIABILITY OF VITRIFIED PORCINE BLASTOCYSTS

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The objective of this study was to improve the vitrification protocol that was used to obtain the first reported litter of pigs resulting from the transfer of vitrified zona pellucida-intact blastocysts (Beebe *et al*. 2000 Theriogenology 53: 249 abstr). We had previously noticed that vitrified and warmed early blastocysts rarely hatched after 48 h in vitro culture, starting to degenerate after 24 h. These experiments examined whether assisted hatching, initially done by removing the zona, then by zona thinning, would improve post-warming in vitro viability. Early blastocysts were surgically recovered from mature Large White x Landrace sows five days after mating. Embryos were washed 3 times in modified phosphate-buffered saline (mPBS; Quinn *et al.* 1982 J. Reprod. Fert. 66:161–168), and then washed twice and cultured in 40-μL droplets of NCSU23 + 10% fetal bovine serum under mineral oil at 39°C until ready for vitrification. Embryos were cultured for 25 min with 7.5 $\mu g\,mL^{-1}$ cytochalasin B, placed into a 0.5-mL eppendorf tube in 30 μL of the culture medium, centrifuged for 13 min at 13000*g* and then recovered back to the culture medium. All media involved in the vitrification protocol included cytochalasin B. Centrifuged embryos were equilibrated in 2 M ethylene glycol in mPBS at 25°C for 5 min, washed briefly in 8 M ethylene glycol + 7% polyvinylpyrrolidone in mPBS for approximately 20 sec, loaded into open pulled straws and plunged into liquid nitrogen. Warming and rehydration was performed by immersing the end of the straw containing the embryos into 1.2 mL 1 M sucrose in mPBS at 39°C, allowing the thawing media to enter the straw and recovering the embryos from the straw with a pipette, followed by 2 min in 1 M ethylene glycol in mPBS, and 2 min in 0.5 M ethylene glycol, both at 25°C. The zona-intact group (ZI) was placed in mPBS at 39°C for at least 5 min, then cultured in vitro for 24 h. The zona-free group (ZF) was washed briefly in mPBS, and the zona was removed with 0.5% pronase in PBS for 30 sec, then washed extensively in mPBS, all at 25°C, and then washed and cultured as described for 24 h. After 24 h embryos that had reformed the blastocoel and expanded were considered viable. Viable embryos were fixed with 100% ethanol and the nuclei stained with 10 $ng\,mL^{-1}$ bisbenzimide, visualized and counted using fluorescent microscopy. There were 3 experiments. Removing the zona did not affect survival rates (ZI 81% $n = 21$; ZF 91% $n = 23$) but improved the cell count by 56% (cell number ± SEM; ZI 39.1 ± 2.8 $n = 17$; ZF 60.8 ± 4.3 $n = 20$; $P < 0.05$ by ANOVA). A subsequent series of experiments found that zona-thinned embryos (0.25% pronase for 10 sec) had the same survival and cell count as zona-free embryos. These experiments show that vitrified porcine blastocysts benefit from assisted hatching, whether the zona pellucida is removed or just thinned.

86 COMPARISON OF TWO VITRIFICATION PROTOCOLS FOR CROSSBRED *BOS INDICUS* × *BOS TAURUS* IN VITRO-PRODUCED EMBRYOS

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Dairy herds in tropical countries are often maintained as crossbred *B. indicus* × *B. taurus* hybrids to take advantage of heterosis, such as resistance to heat stress. Creating crossbred *B. indicus* × *B. taurus* embryos by in vitro methods may offer a means of rapidly improving tropical dairy herds, especially if the embryos can be cryopreserved. The aim of this study was to compare the viability of in vitro-produced crossbred *B. indicus* × *B. taurus* embryos (1/2, 3/4) using two vitrification solutions and equilibration/dilution temperatures. Cumulus-oocyte complexes were aspirated from purebred *B. indicus* and crossbred (*B. indicus* × *B. taurus* hybrid) ovaries, matured in vitro, and fertilized with spermatozoa collected from a Holstein bull. Presumptive zygotes were co-cultured in CR2aa medium with cumulus cells, in a humid atmosphere of 5% CO_2-air at 38.8°C. On day 7 of co-culture, embryos were assessed and classified as good or excellent, and those at the appropriate developmental stage were vitrified using one of two vitrification solutions, a mixture of either glycerol/ethylene glycol (GE) or dimethylsulphoxide/ethylene glycol (DE). Embryos ($n = 34$) assigned to GE vitrification were equilibrated in a medium of PBS + 20% FCS (HM1) containing 10% v/v G for 5 min, followed by 10% v/v G + 20% v/v E for 5 min., and then transferred to a vitrification solution of 25% v/v G + 25% v/v E in HM1 for 30 s. The embryos were immediately aspirated into an Open Pulled Straw (OPS) and plunged into liquid nitrogen. Embryos vitrified in GE were warmed by immersing the OPS in HM1 containing 1 M sucrose for 1 min (37°C), then stepwise diluted in fresh HM1 containing 1 M, 0.5 M, and 0.25 M sucrose for 5 min; and finally washed in HM1. Stepwise equilibration and dilution of GE embryos was at 20°C. Embryos ($n = 43$) assigned to DE vitrification were equilibrated in a medium of PBS + 5% FCS (HM2) containing 10% v/v D + 10% v/v E for 1 min, and then transferred to a vitrification solution of 20% v/v D + 20% v/v E in HM2 for 30 s. The embryos were immediately aspirated into an Open Pulled Straw (OPS) and plunged into liquid nitrogen. Embryos vitrified in DE were warmed by immersing the OPS in HM2 containing 0.25 M sucrose for 1 min (39°C), then stepwise diluted in fresh HM2 containing 0.25 M and 0.15 M sucrose for 5 min, and finally washed in HM2. Stepwise equilibration and dilution of DE embryos was at 39°C. Diluted embryos from both groups and untreated control embryos ($n = 49$) were cultured in TCM-199 with monolayer granulosa cells for 72 h in conditions described above. Blastocyst re-expansion and hatching was assessed and analyzed by chi-square test. Overall, 67% of the thawed embryos were expanded blastocysts (remainder were blastocysts) and 56% were excellent quality (remainder were good). No significant difference ($P > 0.05$) was found between the rates of blastocyst re-expansion and hatching for the GE and DE vitrification procedures (respectively, 59 and 79%, and 41 and 58%). However the hatching rate of control embryos (77%) was significantly higher than that of vitrified embryos ($P < 0.05$). These results indicate that both vitrification procedures are promising for the cryopreservation of crossbred *B. indicus* × *B. taurus* in vitro-produced embryos. Supported by CNPq.

87 ONE-STEP VERSUS TWO-STEP VITRIFICATION AND IN-STRAW DILUTION OF IN VITRO-PRODUCED BOVINE EMBRYOS

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Slow-cooling techniques are widely used in cryopreservation of bovine embryos. We have previously developed a simple, two-step vitrification technique for direct transfer in the field; however, simplification to one-step vitrification would be attractive. Therefore, factorial combinations of two techniques (one-step and two-step) and two post-thaw temperatures until culture (24 and 37°C) were studied. Blastocysts ($n = 220$) sired by two bulls were obtained in vitro in four replicates. Briefly, oocytes were aspirated from 2–8-mm follicles of ovaries obtained at a slaughterhouse, matured, fertilized and cultured in vitro with standard procedures using chemically defined media (CDM1/2 or G1/2). Two-step embryos were transferred in 1 μL into 1 mL of V1-CDM (5 M ethylene glycol (EG) in HEPES-buffered holding medium (HCDM2)), and one-step embryos into a 7-μL droplet of V2-CDM (7 M EG, 0.5 M galactose and 18% w/v Ficoll 70 in HCDM2) for 3 min at 24°C. Next, embryos for the two-step method were moved in 1 μL into a 7 μL droplet of V2-CDM at 24°C. Droplets containing embryos (one or two-step) were loaded into 0.25-mL straws preloaded with a 1-cm column of D-HCDM (0.5 M galactose in HCDM2), then 0.5 cm air, and then 7 cm of D-HCDM followed by 0.5 cm air. The column containing the embryos (0.5 cm (7 μL)) was followed with 0.5 cm air and 1 cm of D-HCDM. Straws were heat-sealed and plunged vertically, sealed end first, into liquid nitrogen just covering the embryo, and the rest of the straw was then slowly immersed. The time from loading to plunging was 40–50 s. Straws were thawed in air (24°C) for 10 s and then in water horizontally at 37°C until ice disappeared. Straws were gently shaken to mix the columns; then, after 5 min at 24 or 37°C, embryos were expelled and cultured in CDM2 + 5% FCS. Re-expansion and hatching rates were evaluated 48 h post thaw. Data (Table 1) were calculated as a percentage of non-vitrified controls for respective replicates (control means: re-expansion 87%; hatching 74%) and analyzed by ANOVA. There were no main effects of post-thawing temperature ($P > 0.1$), indicating that, after thawing, embryos can be kept at room or body temperature. Also, main effect means for re-expansion and hatching for one-step or two-step addition of cryoprotectant were similar ($P > 0.1$), but there was a tendency for higher survival for the two-step procedure. Further refinements of the one-step technique including EG concentrations, embryological stages and equilibration times should be studied.

Table 1. Main effect means (least-square means ± SEM) of vitrified embryos (% of non-vitrified controls)

	Post-thawing temperature (°C)		Vitrification technique	
	24	37	One-step	Two-step
Re-expansion (%)	90 ± 9.8	93 ± 9.8	85 ± 9.8	98 ± 9.8
Hatching (%)	61 ± 13.5	61 ± 13.5	56 ± 13.5	67 ± 13.5

88 ESTABLISHMENT OF PREGNANCIES AFTER VITRIFICATION OF EQUINE EMBRYOS

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Vitrification of equine embryos has been limited, with only one study reporting transfer of vitrified embryos into recipients (1994 Theriogenology 42, 483–488). The objective of this study was to vitrify equine embryos in different stages of development based on a protocol that had been successful in sheep (Naitanas *et al.*, 1996 Theriogenology 46, 813–824) and buffalo (2001 Theriogenology 55, 307). Embryos, Days 6.5 to 7.5, were assigned to a 3 (stage) × 2 (media) factorial design ($n = 3$ per group) based on diameter and developmental stage [(1) morulae + very early blastocysts; (2) blastocysts <300 μm; (3) blastocysts >300 μm] and base media [(1) PBS supplemented with 0.3 mM pyruvate, 3.3 mM glucose and 10% FCS or (2) HCDM (Olson SE and Seidel GE Jr. 2000 J. Anim. Sci. 78, 152–157) with Eagle's essential and nonessential amino acids, 2 mM glucose, 3 mg mL^{-1} BSA and 20% FCS]. Embryos were placed sequentially in 200 μL of 1.4 M glycerol for 5 min, 200 μL of 1.4 M glycerol + 3.6 M ethylene glycol for 5 min, and 30 μL of 3.4 M glycerol + 4.6 M ethylene glycol. Within 30 s, the 30-μL drop containing the embryo was loaded into the center of a 0.25-mL straw separated by air from columns (30 μL) of the same solution. Straw ends were loaded with columns (60 μL) of 0.5 M galactose in base medium. The straw was heat-sealed and exposed to liquid-nitrogen vapor for 1 min before being plunged into liquid nitrogen. To warm embryos, straws were held in air for 10 s and then immersed in 20°C water for 10 s. Embryos were transferred into 200 μL of 0.25 M galactose solution for 5 min, and then placed in PBS or HCDM. Within 10 min, embryos ($n = 3$ per recipient), grouped for stage and media, were transferred nonsurgically into the uteri of 6 recipients that had ovulated 6 days earlier. Ultrasound exams were done at 4, 6, 8 and 10 days after transfer. Three morulae or very early blastocysts and one blastocyst <300 μm, all vitrified in PBS, formed embryonic vesicles. Two of 3 vesicles, in the same recipient, were manually removed on Day 16. The remaining vesicle and the single vesicle, from a blastocyst <300 μm, had normal development until Day 38 when pregnancies were terminated. No pregnancies were observed on Day 16 after use of HCDM. Because numbers of embryos were low, it is not clear if HCDM had a detrimental effect ($P > 0.1$). Embryos were transferred in October, at the end of the breeding season. Therefore, quality of available recipients could have affected pregnancy rates. No pregnancies were observed from expanded blastocysts. Potentially, the embryonic capsule, present at this stage, impeded diffusion of cryoprotectants. These preliminary results demonstrate the potential for vitrification of small equine embryos.

89 EFFECT OF PRE-EQUILIBRATION PROCEDURES ON THE DEVELOPMENT POTENTIAL OF VITRIFIED BOVINE OOCYTES AFTER IVF

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This experiment was designed to improve oocyte developmental potential after droplet vitrification and IVF by different equilibration procedures as reported by Papis K. *et al.* (2000 Theriogenology 54, 651–658). Bovine oocyte-cumulus complexes were collected from slaughterhouse ovaries, and matured in vitro for 24 hours in TCM199 medium supplemented with 7.5% FBS, 0.5 $\mu g\,mL^{-1}$ FSH, 5 $\mu g\,mL^{-1}$ LH and 2 $\mu g\,mL^{-1}$ estrodial under 5% CO_2 in humidified air at 39°C. Oocytes were then partially stripped of most expanded cumulus cells with only 3–5 inner layers left by a short exposure to 0.1% hyaluronidase and careful pipetting. Oocytes were randomly assigned to the following pre-equilibration treatments (39°C): Group 1, oocytes were pre-equilibrated in medium 1 consisting of holding medium (HEPES-buffered TCM199 supplemented with 20% FBS) + 10% ethylene glycol (EG) (v/v) + 10% dimethylsulphoxide (DMSO) (v/v) for 30–45 s; Group 2, oocytes were pre-equilibrated in medium 1 for 3 min; and Group 3, oocytes were pre-equilibrated in medium 2 (holding medium + 3% EG + 3% DMSO) for 20 min. Oocytes were then equilibrated in 1.0 mL of vitrification medium (holding medium + 20% EG and 20% DMSO) as described by Vajta G *et al.* (1998 Mol. Reprod. Dev. 51, 53–58). Each droplet contained 8–10 oocytes in about 2 μL vitrification medium and was dropped into liquid nitrogen immediately after 25–30 s exposure to the vitrification solution. Vitrified oocytes were subsequently warmed by transfer into 3 mL holding medium containing 0.25 M sucrose (39°C). After standard BO IVF procedure, presumptive zygotes were cultured in CR1-aa medium supplemented with 6 $mg\,mL^{-1}$ BSA at 39°C in a humidified atmosphere of 5% CO_2, 5% O_2 and 90% N_2 for eight days. Non-vitrified oocytes were used as a control. Data were analyzed with General Linear Model, SPSS 11.0. As shown in Table 1, there was a significant low survival rate ($P < 0.05$) in Group 3 compared to other treatments. The cleavage rates of Groups 1 and 2 were significantly ($P < 0.05$) higher than that of Group 3, but lower than that in the Control. Furthermore, blastocyst rate on Day 8 in Group 2 was significantly ($P < 0.05$) higher than in the other two experimental groups, but still lower than the Control. This study suggests that the developmental capacity of vitrified oocytes can be improved by a duration of equilibration for 3 minutes in holding medium plus 10% EG and 10% DMSO via droplet vitrification.

Table 1. In vitro development of vitrified bovine oocytes following warming and IVF

Treatment	Total No. oocytes	No. (%) Survived	No. (%) Cleaved	No. (%) BL Day 7	No. (%) BL Day 8
Group 1	180	154 (85.30)[a]	116 (64.32)[a]	20 (11.01)[a, d]	31 (17.11)[a]
Group 2	187	169 (90.41)[a]	112 (59.98)[a]	31 (16.54)[a]	49 (26.07)[b]
Group 3	169	120 (70.51)[b]	68 (39.84)[b]	12 (6.96)[b, d]	14 (8.24)[c]
Control	417	–	333 (79.84)[c]	201 (48.22)[c]	235 (56.37)[d]

[a, b, c, d] values within columns with different superscripts are significantly different ($P < 0.05$). BL = blastocysts.

90 EFFECTIVE VIABILITY THRESHOLD FOR PRESERVED HONEY BEE SEMEN

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The honey bee, *Apis mellifera*, is one of very few insects for which artificial insemination is possible, and preservation of semen has been attempted. Honey bee queens normally mate with 7–20 males early in life, store the semen in the spermatheca and release approximately 30 sperm to fertilize each egg. Fertilized eggs are females (queens or workers) and unfertilized eggs are males (drones). The queen controls release of spermatozoa, laying only worker eggs across large areas of comb, and drone eggs in small separate groups. As stored spermatozoa are depleted, a queen will begin to lay mixed groups of workers and drones. Semen cryopreserved following existing protocols (Harbo JR 1983 Annals Entomol. Soc. Amer. 76, 890–891) has less than 20–25% live spermatozoa (based on percentage of workers reared). This viability level is insufficient to successfully fertilize all of the 1000–1500 eggs per day from an active queen, and the colony slowly dwindles and dies. Using dual fluorescent staining (Collins AM and Donoglye AM 1999 Theriogenology 51, 1513–1523). I have determined that semen with 46% live spermatozoa (50% fresh and 50% freeze-killed semen v/v) or more, produces consistent laying patterns with all fertilized eggs (workers) from inseminated queens (Collins AM 2000 Apidologie 31, 421–429). The current study was done to determine how long queens inseminated with mixed fresh:frozen semen would continue to produce normal brood (collective term for all eggs, larvae and pupae), as compared to those inseminated with all fresh semen, or naturally mated in flight. In spring, sister queens were reared and inseminated with [1] all fresh semen, [2] half fresh and half freeze-killed semen, or [3] ¼ fresh and ¾ freeze-killed semen, or [4] were allowed to mate naturally. The queens were evaluated monthly in individual colonies for percentage of worker offspring v. drone offspring and area of comb with eggs, larvae and pupae, until the queen failed. As expected, the inseminated queens produced less brood than did the naturally mated [4] queens. All of the queens with only fresh semen [1] produced normal brood with 100% workers, and lived well into the winter. Eighty-eight percent of the queens inseminated with half freeze-killed semen [2] performed as well, although some of those failed within a few months. The remaining 22% began laying with mixed worker and drone brood. In treatment [3], 60% of the queens produced variable but high levels of drones in reduced areas of egg laying. Those queens in this group that did lay well (40%) also survived into the winter. These results mean that preserved semen that has 50% or better viable sperm has a good probability of producing inseminated queens that will lay normally for a complete beekeeping season. This is sufficient time for breeders to rear daughter queens from such matings, and incorporate desirable genotypes into a breeding program.

91 IMPACT OF PROPYLENE GLYCOL V. ETHYLENE GLYCOL ON OOCYTE MATURATION, SPINDLE INTEGRITY, FERTILIZATION AND EMBRYO DEVELOPMENT IN VITRO IN THE DOMESTIC CAT

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A thorough characterization of cryoprotectant (CPA) sensitivity is required to formulate a successful cryopreservation protocol for any biomaterial. The aim of this study was to characterize the toxic impact of various CPA types, concentrations, and exposure temperatures on the immature domestic cat oocyte. In Experiment 1, grade I immature oocytes ($n = 561$) were exposed (30 min; 25°C or 0°C) to 0 M, 0.75 M, 1.5 M, or 3 M of propylene glycol (PrOH) or ethylene glycol (EG) in PBS + 20% fetal calf serum (v/v). After exposure, CPA was removed step-wise by subjecting oocytes to decreased CPA concentrations. Oocytes were cultured (30 h; 38.5°C, 5% CO_2) in IVM medium as reported previously (Wolfe and Wildt 1996 J. Reprod. Fertil. 106, 135–141). Oocytes were then fixed and stained to examine nuclear status (Hoechst 33342) and spindle integrity (FITC-labeled anti-α-tubulin antibodies; Sigma Chemical Co., St. Louis, MO). Experiment 2 was designed on the basis of Experiment 1 results to assess the impact of the spindle abnormalities on subsequent embryo development. Oocytes ($n = 776$) were exposed to CPA conditions yielding optimal nuclear maturation with either high (0.75 M or 3.0 M PrOH or 1.5 M EG at 25°C) or low (1.5 M PrOH at 25°C) proportions of abnormal spindle. After IVM, oocytes were inseminated with thawed semen (5×10^5 motile sperm mL^{-1}) in Ham's F-10 (Irvine Scientific, St-Anna, CA). At 16 h post-insemination, oocytes were fixed and stained (Hoechst 33342) to assess IVF success (pronuclear formation) or cultured in vitro for 7 days to assess embryo development. Data were analyzed by ANOVA and Tukey's multiple comparison test. In Experiment 1, CPA treatment had no effect (NS) on meiotic progression to metaphase I. However, percentage of oocytes reaching metaphase II (MII) was reduced ($P < 0.05$) in 3.0 M PrOH at 0°C (29.3 ± 8.3%; mean ± SD), 3.0 M EG at 25°C (33.7 ± 8.9%), and 0°C (29.4 ± 11.0%) compared to all other conditions examined (range, 52.0% to 62.0%). All CPA treatments also increased ($P < 0.05$) spindle abnormalities at MII (range, 40.3% to 75.9%) compared to control (13.8 ± 8.6%), except 1.5 M PrOH at 25°C (20.7 ± 10.1%). None of the CPA treatments in Experiment 2 influenced IVF success (range, 55% to 63%; NS). However, percentage of cleaved embryos was reduced ($P < 0.05$) in 0.75 M PrOH (32.1 ± 4.1%), 1.5 M EG (33.4 ± 4.0%), and 3.0 M PrOH (29.3 ± 3.8%) compared to control (50.1 ± 4.0%) or 1.5 M PrOH (50.6 ± 4.9%). Developmental competence (number of blastocysts relative to number of cleaved embryos) also was impaired ($P < 0.05$) in 1.5 M EG (16.5 ± 7.4%) and 3.0 M PrOH (14.9 ± 7.8%) compared to the other conditions (range, 32.5% to 38.5%), including 1.5 PrOH at 25°C (32.5 ± 7.8%). In conclusion, exposure of immature oocytes to 1.5 M PrOH at 25°C does not adversely impact oocyte maturation, MII spindle, fertilization, or embryo development in vitro in the domestic cat.

92 THE ADDITION OF SEMINAL PLASMA FROM INDIVIDUAL BOARS TO FREEZING EXTENDER CAN IMPROVE THE POST-THAW SPERM SURVIVAL

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Contradictory results have been reported about the effect of seminal plasma (SP) on the freezability of mammalian spermatozoa. In pigs, current methods for sperm cryopreservation involve removing seminal plasma. Therefore, no conclusive evidence of the potential effect of SP on the freezability of boar spermatozoa has been reported. In this study, we evaluate the effect of the addition of low concentrations of SP from individual boars to the freezing extender on post-thaw sperm survival. Sperm cryopreservation procedure included: dilution of sperm-rich fraction in Beltsville Thaw Solution extender (BTS), cooling to 17°C for 16 h, centrifugation at 2400*g* for 3 min, dilution in lactose/egg-yolk/glycerol/Equex Stem (freezing extender) to a final concentration of 1×10^9 sperm mL^{-1}, dispensing into 0.5-mL straws, and freezing in a programmable cell freezer at 20°C min^{-1}. Thawing was carried out in a waterbath at 37°C for 20 s. Post-thaw sperm survival was assessed by progressive sperm motility (PSM) using a CASA system (SCA); plasma membrane integrity (PMI) and acrosome membrane integrity (AMI) were assessed by flow cytometric procedures (SYBR-14/PI and FITC-PNA/PI, respectively) at 30 and 150 min post-thawing in BTS-diluted thaw spermatozoa held in a waterbath at 37°C. Four individual seminal plasma donors (SP1 to SP4) were selected in a preliminary study in which 48 ejaculates from 12 boars (4 ejaculates/boar) were cryopreserved. Then the boars were classified into 3 groups (good, moderate and bad freezers) based on their post-thaw sperm survival. SP1 and SP2 were good freezers (>60% PSM and PMI), SP3 was a moderate freezer (40–60% PSM and PMI) and SP4 was a bad freezer (<40% PSM and PMI). In the main experiment, pooled sperm-rich fractions collected from 9 mature hybrid boars were divided into five aliquots and each was diluted with freezing extender supplemented with 0% (control) or 10% of SP (1–4). Data from eight replicates were analyzed as a split plot design using a PROMIXED model. The addition of SP to freezing extender had a significant effect ($P < 0.05$) on post-thaw sperm survival compared to control. Moreover, there were significant differences ($P < 0.05$) between SP donors. PSM, PMI and AMI were significantly ($P < 0.05$) higher in SP1 (56.71 ± 4.30; 57.16 ± 4.01 and 57.22 ± 4.01, respectively) and SP2 (59.48 ± 4.30; 60.17 ± 4.01 and 60.05 ± 4.01, respectively) compared to control (50.39 ± 4.30; 49.98 ± 4.01 and 49.54 ± 4.01, respectively). There were no differences ($P > 0.05$) between SP3, SP4 and control. These results indicate that the addition of SP from particular boars (good freezers) to freezing extender may improve post-thaw sperm survival. Individual differences in the SP composition should explain the above results. Supported by INIA (RZ01-019) and MCYT (AGL2001-0471).

93 COMPUTER-ASSISTED ANALYSIS OF BOVINE SPERM MOTILITY BEFORE AND AFTER CRYOPRESERVATION

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Sperm cryopreservation causes various types of damage, including membrane injury, oxidative stress, and loss of the acrosome. In cattle, the mortality rate after sperm cryopreservation reaches roughly 50%, and surviving sperm cells have a lower motility and lower fertility than their fresh counterparts. Large variations are also observed between bulls. The aim of this study was to analyse different motility parameters before and after

freezing in order to establish correlations. The final objective is to determine, before freezing, parameters that could predict the characteristics of motility after freezing. A computer-assisted sperm analyser (Hobson Sperm Tracker) was used. We analyzed one ejaculate from 30 different bulls before and after freezing (minimum 300 spermatozoa/analysis). Reliable parameters (<10% variation for the same ejaculate) were then selected and included VCL (curvilinear velocity), VAP (average path velocity), MAD (mean angular head displacement), ALH (amplitude of lateral head displacement), STR (straightness of path), and the percentage of motility (%Mot). Linear regressions were established between those parameters before and after freezing. Results are shown in Table 1. The velocity parameters (VAP, VCL, and STR) of the motile sperm were conserved after freezing. Moreover, ejaculates with a high proportion of motile sperm before freezing have, on average, better values for velocity parameters after freezing, while no correlation was found between the percentage of motile sperm before and after freezing. The only parameter of fresh sperm that seems to be correlated with the proportion of motile sperm cells after freezing is MAD (inverse correlation). This could mean that an ejaculate with a high proportion of spermatozoa showing important lateral displacements of the head is more sensitive to cryopreservation. Similarly, a high MAD before freezing was related to a low velocity after thawing. A high MAD could result from a high proportion of capacitated spermatozoa, which is detrimental to their survival and motility. In conclusion, few parameters related to the motility can predict the proportion of motile sperm after freezing. However, by combining several parameters, it seems possible to predict the characteristics of motility of the sperm. Although further investigations are needed, the present evaluation could be of interest to evaluate the freezability of ejaculates, to understand variations between bulls, or to set up new freezing protocols.

Table 1. Coefficients of correlation (r^2) before and after freezing, calculated from 30 ejaculates from 30 different bulls

Pre Post	%Mot	VCL	VAP	STR	MAD	ALH
%MOT	0.11	0.28*	0.29*	0.28*	0.12(–)	0.02(–)
VCL	0.03	0.31*	0.40*	0.11	0.18(–)*	0.28(–)*
VAP	0	0.43*	0.52*	0.18*	0.20(–)*	0.20(–)*
STR	0	0.13*	0.33*	0.30*	0.35(–)*	0.32(–)*
MAD	0.17(–)*	0.19(–)*	0.27(–)*	0.25(–)*	0.12	0.03
ALH	0	0.07(–)	0.21(–)*	0.26(–)*	0.26*	0.33*

(–) inverse correlation. *Significantly correlated (linear regression—$P < 0.05$).

94 MEIOSIS INHIBITION OF BOVINE OOCYTES: EFFECTS ON SURVIVAL AFTER VITRIFICATION BY OPEN PULLED-STRAW METHOD

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Mammalian oocytes remain one of the most difficult cell types to successfully cryopreserve. The in vitro-maturation protocols (IVM) have a large impact on the oocyte maturation. Consequently, inhibition of meiosis has been used to improve developmental competence of oocytes without reducing blastocyst rates. Moreover, the meiotic stage influences the ability of oocytes to survive cryopreservation. This work analyzes the effect of the inhibition of meiosis (prematuration) on the freezability of the bovine oocyte. Cumulus-oocyte complexes (COCs) were recovered from slaughterhouse ovaries. Inmature oocytes (I) with compact cumulus and evenly granulated cytoplasm were selected. Prematuration (PM) was performed by incubating COCs for 22 h in TCM199 $NaHCO_3$ and roscovitine 25 μM. IVM was accomplished in TCM199 $NaHCO_3$, 10% FCS, FSH-LH and 17β-estradiol. Oocytes were subjected to 5 treatments prior the vitrification (see table). COCs were partially denuded from cumulus cells and vitrified/warmed using the OPS system (Vajta *et al.* 1998 Mol. Reprod. Dev. 51, 53–58). Warmed oocytes were fertilized (Day 0) and presumptive zygotes having a normal morphological appearance were cultured in SOFaa + 5% of FCS (Day 3); elements with degenerated appearance were discarded and recorded. Fresh oocytes submitted to IVM (c-M) or prematured and matured (c-PM + M) were fertilized and cultured as controls. Data were analyzed by ANOVA and Duncan's multiple range test and expressed as LSM ± SE. Developmental data are referred to the zygotes cultured. Only oocytes vitrified after IVM reached the blastocyst stage, but at lower rates than fresh controls. However, no differences were found between treatments at any developmental stage. Oocytes vitrified both as prematured + matured and immature oocytes showed increased proportions ($P < 0.01$) of degenerated oocytes (37.3 ± 5.9 and 49.9 ± 5.9, respectively), as compared with oocytes matured before vitrification (17.6 ± 5.9). These results show that effects induced by incubation with roscovitine (Lonergan *et al.* 2003 Mol. Reprod. Dev. 64, 369–378) in combination with cryodamage compromise the oocyte developmental ability. Supported by CICYT, AGL2001-379.

Treatment		N	R	% Cleaved	% Blastocysts	
Before OPS[a]	After Warming[b]			Day 3	Day 7	Day 8
I	M	139	6	25.4 ± 5.2^{x}	0.0^{x}	0.0^{x}
I	PM+M	107	6	11.5 ± 5.2^{x}	0.0^{x}	0.0^{x}
PM	M	126	6	28.2 ± 5.9^{x}	0.6 ± 3.5^{x}	0.8 ± 3.1^{x}
PM+M	None	132	6	25.8 ± 5.2^{x}	0.0^{x}	0.0^{x}
M	None	142	6	37.0 ± 5.2^{x}	1.5 ± 3.2^{x}	1.5 ± 2.8^{x}
c-M[c]	None	238	7	88.7 ± 5.2^{y}	36.4 ± 3.2^{y}	37.7 ± 2.8^{y}
c-PM+M[c]	None	156	7	84.4 ± 5.2^{y}	27.5 ± 3.2^{y}	32.3 ± 2.8^{y}

[a]Oocyte status before OPS (I: inmature; PM: prematured; M: matured); [b]Oocyte treatment after warming; [c]Unfrozen control. N: number of zygotes cultured; R: replicates; [x,y]Differing superscripts express significant differences ($P < 0.01$).

95 FIRST REPORT ON CLEAVAGE DEVELOPMENT FOLLOWING CRYOPRESERVATION OF ADULT AND PREPUBERTAL RHESUS MONKEY (*MACACA MULATTA*) OOCYTES

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Cryopreservation of non-human primate oocytes would allow a more efficient management of biological resources for medical research and endangered species preservation. Despite previous attempts, no cleavage-stage development has been reported following cryopreservation of rhesus monkey (*Macaca mulatta*) or other non-human primate oocytes. The extreme chilling sensitivity of rhesus monkey oocytes (Songsasen N *et al.*, 2002 Fertil. Steril. 77, 818–825) might be overcomed by vitrification methods. Our aim was to test the Solid Surface Vitrification (SSV, Dinnyes A *et al.*, 2000 Biol. Reprod. 63, 513–518) method on metaphase II (MII)-stage rhesus monkey oocytes and to achieve successful fertilization of warmed oocytes. Oocytes were obtained from hormonally stimulated adult and unstimulated prepubertal females and matured in vitro for 36 to 48 h as described by Zhang P *et al.* (2001 Biol. Reprod. 64, 1417–1421). The vitrification solution contained 35% ethylene glycol (EG), 5% polyvinyl pyrrolidone, and 0.4 M trehalose in Tyrode-lactate (TL)-HEPES medium with 3 mg mL^{-1} BSA added. Oocytes at MII-stage were equilibrated in 4% EG in TL-HEPES at 37°C for 10 to 12 min and then exposed to the vitrification solution at 37°C for about 20 s. Oocytes in 2-μL droplets of vitrification solution were dropped onto a metal surface at about −180°C where they were vitrified instantaneously. Warming was performed by moving the vitrified droplets into 0.3 M trehalose at 37°C. Recovered oocytes were exposed to 0.15 M and then 0.075 M trehalose for 2 min each and then rinsed three times in TL-HEPES. Warmed oocytes were fertilized in vitro and then cultured for 96 h in 50-μL drops of mCMRL-1066 containing 20% FCS at 37°C in a humidified atmosphere of 5% CO_2, 5% O_2, and 90% N_2, as described in details by Zhang (see above). A total of 21 MII oocytes were collected from 2 adult and 55 MII oocytes from 14 prepubertal animals. No difference has been found between the rate of adult-origin (15/19; 79%) v. prepubertal-origin (37/52; 71%) oocytes surviving the vitrification process without lysis ($P > 0.05$, chi-square analyses). Following subsequent IVF, 1/15 (7%) adult-origin and 4/23 (17%) prepubertal-origin oocytes cleaved further, which was lower than that of the corresponding controls (1/2 (50%) and 1/3 (33%), respectively). The furthest development observed following cryopreservation was 16-cell stage, verified by counting of stained nuclei. This is the first report on cleavage-stage development of cryopreserved rhesus monkey oocytes, demonstrating the feasibility of vitrification and the potential for gene banking of non-human primate oocytes, even from prepubertal animals. Further experiments are needed to achieve higher rates of cryosurvival and progeny development. This research was supported by a Chinese-Hungarian Bilateral Technological and Scientific Collaboration Project (No. CHN14/02).

96 ULTRASTRUCTURE AND CELL DEATH OF VITRIFIED PORCINE BLASTOCYSTS

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Cryopreservation of porcine embryos by the simple open pulled straw (OPS) method was recently reported to result in live offspring (Berthelot *et al.*, 2000, Cryobiology 41; 116–124,) and is evaluated in the present study by light (LM) and transmission electron microscopy (TEM) as well as by TUNEL staining in order to detect morphological and molecular signs of cell death and subsequent regeneration. Blastocysts were collected from gilts on Day 5 (Day 0 = 1st AI) and were randomly assigned to one of three groups: Fresh controls (FC) were fixed immediately after collection, and vitrified embryos were fixed either immediately after vitrification and warming (V0) or after 24 h of culture upon warming after vitrification (V24). In each of the three groups, embryos were fixed and processed for LM/TEM (FC, $n = 13$; V0, $n = 20$; V24, $n = 18$) or TUNEL staining (counter staining with propidium iodide) and confocal laser scanning microscopy (FC, $n = 32$; V0, $n = 31$; V24, $n = 33$). At LM, the FC embryos displayed a well-defined trophoblast (Tb) and inner cell mass (ICM), expanded blastocoele cavity and a narrow or no perivitelline space. In V0 embryos, collapse of the blastocoele cavity and cell swelling was detected. At the TEM level, the V0 embryos showed extensive injuries including a general distension or shrinkage of mitochondria and massive increase in the amount of membrane-bound vesicles, vacuoles and secondary lysosomes. In both FC and V0 embryos, the presence of dead or phagocytozed cells in the ICM and Tb was occasional. A few extruded cells were often noticed in the perivitelline space or in the blastocoele cavity, and such cells ranged from being rather normal to showing typical morphological features of apoptosis. TUNEL staining confirmed the presence of a few apoptotic cells in both groups of embryos. Approximately 2/3 of the V24 embryos had, as evaluated by LM, partially recovered, re-expanded or even hatched whereas the remaining 1/3 had degenerated. At the TEM level, the recovered embryos displayed almost normal blastocyst morphology, except for a widening of the perivitelline space, accumulation of debris, increased electron-lucidity of the ICM and partial distension of mitochondria. The degenerated embryos had disintegrated into a poorly defined mass of cells and debris including cells with either decreased or increased electron-density of the cytoplasm and with abundant degeneration of mitochondria and other organelles. Both recovered and degenerated embryos displayed persistent abundant presence of small membrane-bound vesicles, vacuoles and secondary lysosomes. All V24 embryos displayed increased occurrence of dead or phagocytozed cells in the ICM and Tb as well as increased occurrence of extruded cells showing typical morphological features of apoptosis or secondary necrosis. TUNEL staining confirmed the increased occurrence of apoptotic cells in this group of embryos. In conclusion, immediately after vitrification and warming, porcine embryos displayed severe subcellular damages, but during 24 h of culture the majority of the embryos were able to regenerate. Along with the regenerative process, apoptosis became evident. Supported by CRAFT EC contract no. QLK5-CT-2002-70983.

97 EFFECT OF RAPID TEMPERATURE CHANGE ON VIABILITY OF FROZEN-THAWED IVM/IVF BOVINE EMBRYOS

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In on-farm conditions, frozen bovine embryos are frequently thawed at various environmental temperatures. Thawing temperature is an important factor affecting the viability of frozen-thawed bovine embryos. The present study investigated the effects of rapid temperature change on the viability of frozen IVM/IVF bovine embryos after thawing. Day 7- and 8- (Day 0 = day of insemination) expanded blastocysts were used in this study. Embryos were produced as previously described by Hamano & Kuwayama (1993 Theriogenology 39, 703–712,). Embryos were frozen in TCM-199 supplemented with 1.4 M glycerol, 20% calf serum (CS), and 0.25-M sucrose. The embryos were loaded into 0.25 mL straws. After equilibration, the straws were placed directly into a precooled alcohol chamber of a freezer at −6°C, seeded 1 min later, held at −6°C for 10 min, cooled to −25°C at a rate of 0.33°C/min, and then plunged into liquid nitrogen. Embryos were thawed by holding the straws in room temperature air for 10 s, and then immersing them in a 35°C water bath for 10 s. The thawed straws were randomly assigned to one of two groups. Some thawed straws were held for 5 min at −15, −5, 0, 5, or 15°C, and were then transferred directly into a water bath at 35°C for 5 min (Group 1). The remaining straws were subjected to the same post-thaw cooling step procedures as Group 1 two times (Group 2). The embryos were then directly rehydrated in PBS supplemented with 5% CS at 35°C, and cultured in TCM-199 supplemented with 5% CS and 0.1 mM β-mercaptoethanol. The morphology and hatching of embryos was assessed 72 h later. Data were analyzed using the chi-square method and Fisher's exact test. The results are presented in the Table. There were no significant differences in the hatching rate among 5 temperatures in Group 1. Although there were no differences in the hatching rate of embryos held at −5, 0, 5, or 15°C after thawing, the rate for embryos held at −15°C was significantly lower than those of the other treatments in Group 2 ($P < 0.05$). The straws held at −15°C twice (Group 2), showed refreezing. These results suggest that exposing thawed straws to a broad range of environmental temperatures (−5 to 15°C) had no effect on the viability of frozen-thawed IVM/IVF bovine embryos. However, embryos might be irreversibly damaged when held at −15°C.

Table 1. Effect of rapid temperature change on the viability of frozen-thawed IVM/IVF bovine embryos

Temperature (°C)	No. of embryos	No. of hatching blastocysts (%) at 72 h	
		Group 1	Group 2
−15	40	36 (90)[a]	23 (57.5)[b]
−5	40	39 (97.5)[a]	40 (100)[a]
0	40	38 (95)[a]	39 (97.5)[a]
5	40	37 (92.5)[a]	34 (85)[a]
15	40	34 (85)[a]	34 (85)[a]
Control	40	37 (92.5)[a]	

[a, b]Values with different superscripts are significantly different ($P < 0.05$).

98 CONCEPTION RATES USING BRAHMAN BULL SEMEN FROZEN IN MILK BASED EXTENDER CONTAINING EGG YOLK OR SOYBEAN LIPIDS; A FIELD STUDY IN A TROPICAL ENVIRONMENT

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The objective of this study was to examine the substitution of soybean-origin phospholipids for egg yolk in Brahman bull semen extender. Semen was frozen in 3 different low-fat milk (1%) based extenders containing 10 mg mL^{-1} of fructose and supplemented with: 8% of whole egg yolk (Extender 1, control), 8% rectified egg yolk (egg yolk granules were removed by double centrifugation at 3000*g* for 1 h at 5°C; Extender 2), and 7.3 mg mL^{-1} of phospholipids of soybean-origin containing 10% of phosphatidyl choline (Extender 3). All 3 extenders were supplemented with 1000 IU of penicillin, 1 mg mL^{-1} streptomycin and 150 μg mL^{-1} lincomycin. The semen was collected by means of artificial vagina from 3 Brahman bulls, and AI was performed during the dry season between December and April in a tropical forest environment. The mean temperature for the region was 26–30°C, with mean rainfall of 900–1500 mm/year and the relative humidity of 60–70%. Ejaculates with at least 60% motility were diluted in 2 steps as follows: in step 1, each ejaculate was split into 3 even parts and diluted at 26°C with each of the extenders containing no glycerol, and in step 2, 14% of glycerol was added in 15-minute intervals to a final glycerol concentration of 7%. Semen was aspirated into 0.5 mL plastic straws (20×10^6 sperm/per straw), frozen 7 cm above liquid nitrogen (LN_2) for 8 min, and then plunged into LN_2. Straws were thawed in a water bath at 37°C for 30 s. Each experiment was replicated 3 times (different collection days). Sperm viability was tested within artificial insemination trials. Results are based on the pregnancy rates of crossbreed Brahman cows determined by palpation 45 Days after AI and by calving rates. Data were compared by chi-square analysis. In Experiment I, a total of 157 cows were inseminated with semen collected from 3 different bulls (A, B and C) and frozen in 3 different extenders (1, 2 and 3; 3 × 3 factorial design). Bull A, Extender 1, 2 and 3 ($n = 19$, 20 and 22); Bull B, Extender 1, 2 and

3 ($n = 20$, 20 and 20) and Bull C, Extender 1, 2 and 3 ($n = 22$, 15 and 24), respectively. Although semen from all 3 bulls frozen in Extenders 2 and 3 fostered numerically higher pregnancy rates (from 30% for Bull B and Extender 2 to 50% for Bull C and Extender 3) than in Extender 1 (from 23.5% for Bull C to 40% for Bull B), there were no differences ($P < 0.05$) between bulls with any of 3 extenders on the pregnancy rates. In Experiment II, a total of 117 cows were inseminated with semen collected from Bull B and frozen in Extender: 1 ($n = 37$), 2 ($n = 48$) and 3 ($n = 39$). There were significantly higher ($P < 0.05$) calving rates for cows inseminated with semen frozen in Extender 2 and 3 (41.6% and 46.1%, respectively) than in Extender 1 (24.3%). It can be concluded that rectified egg yolk may improve viability of frozen semen, and that phospholipids of soybean origin can be successfully substituted for egg yolk in Brahman bull milk based semen extender. Supported by Bioniche Inc, Belleville, Ontario, Canada

99 MACROMOLECULES FOR VITRIFYING BOVINE OOCYTES

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Vitrification of oocytes would make them available for research and clinical purposes wherever and whenever needed. However, development rates and quality of blastocysts arising from vitrified oocytes have been low. Zona hardening following exposure to vitrification solutions and cooling could contribute to low fertilization rates. Addition of fetal calf serum (FCS) to handling media and vitrification solutions can prevent zona hardening, but FCS may be detrimental to resulting embryos and spread viral diseases, and its composition varies among batches. Fetuin is the component responsible for the protective effect of FCS (Landim-Alvarenga FC *et al.*, 2002 Anim. Reprod. Sci. 71, 181–191). Our objective was to determine whether fetuin is a suitable substitute for FCS during vitrification. Oocytes derived from slaughterhouse ovaries were matured in a chemically defined medium with hormones and with or without 1 mg mL^{-1} fetuin at 39°C in 5% CO_2 in air. At 22 h after the start of maturation, oocytes were transferred to one of the following handling media: 2% BSA, 2% BSA + 1 mg mL^{-1} fetuin, or 20% FCS in TCM-199 + HEPES (HTCM-199). In the same media plus 100 IU mL^{-1} hyaluronidase, cumulus cells were partly removed by gentle pipetting. Oocytes with approximately 3 layers of cumulus were chosen for two-step vitrification. First, they were exposed to VS1 (10% ethylene glycol (EG), 10% DMSO, 6% PVP in HTCM-199) for 30 s, then to VS2 (20% EG, 20% DMSO, 6% PVP, 0.48 M galactose in HTCM-199) for 25 s, loaded into cryoloops in groups of five, and plunged into liquid nitrogen. Rapidly warmed oocytes were moved stepwise in 0.5, 0.25, 0.125, and 0 M galactose in HTCM-199 + 20% FCS, 3 min each. All procedures were conducted at 39°C. Warmed oocytes were placed in maturation medium for an additional hour, and then fertilized and cultured according to standard procedures (Olson SE and Seidel GE Jr 2000 J. Anim. Sci. 78, 152–157). About 2000 oocytes were used in 11 replicates with semen of 4 bulls. The experimental treatments and controls were: A: maturation BSA, handling BSA; B: maturation BSA, handling FCS; C: maturation BSA, handling BSA + fetuin; D: maturation BSA + fetuin, handling BSA + fetuin; E: non-vitrified control, maturation BSA; F: non-vitrified control, maturation BSA + fetuin (Table 1). Controls did not differ ($P > 0.1$) from each other, nor were there differences among vitrification treatments. Controls resulted in greater cleavage and more 8-cell embryos and blastocysts than vitrified treatments, and also tended to have higher cell numbers. In summary, fetuin can replace FCS during handling without decreasing the success of vitrification.

Table 1. Development of vitrified oocytes and controls

Treatment	% cleavage	% 8-cell	% bl D8*	% bl D9*	Cell no.
A	59 (±4)^{b}	50 (±4)^{b}	6 (±3)^{b}	5 (±3)^{b}	80 (±17)
B	63 (±4)^{b}	54 (±4)^{b}	7 (±3)^{b}	11 (±3)$^{b, c}$	100 (±14)
C	65 (±4)^{b}	55 (±4)^{b}	6 (±3)^{b}	7 (±3)^{b}	95 (±18)
D	62 (±5)^{b}	47 (±5)^{b}	7 (±3)^{b}	10 (±3)$^{b, c}$	unestimated
E	82 (±5)^{a}	70 (±4)^{a}	27 (±3)^{a}	25 (±3)^{a}	127 (±14)
F	84 (±5)^{a}	71 (±5)^{a}	27 (±3)^{a}	23 (±4)$^{a, c}$	123 (±16)

$^{a, b, c}$ Means without common superscripts differ significantly ($P < 0.01$), ANOVA of arc sine transformed data. LS means ± SE. *% Blastocysts on Day 8 or Day 9.

100 EFFECTS OF USING MICRO-PIPETTE TIPS OF DIFFERENT DIAMETERS AND VOLUME OF VITRIFICATION SOLUTION ON THE VIABILITY OF IVM BOVINE OOCYTES AFTER VITRIFICATION

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The objective of this study was to investigate the effects of the diameters of micro-pipette tips and the volume of vitrification solution (VS) on viability of IVM bovine oocytes after vitrification. COCs were aspirated from 2–5 mm follicles of ovaries obtained at a local abattoir. COCs were matured for 19 h in TCM-199 supplemented with 5% calf serum (CS) and 0.02 mg mL^{-1} FSH at 38.5°C in an atmosphere of 5% CO_2 in air. The matured oocytes were then vitrified on the basis of Kuwayama and Kato (2000 J. Assist. Reprod. Genet. 17, 477 abst). Matured oocytes were first exposed to 7.5% ethylene glycol (EG) and 7.5% DMSO in holding medium (HM; Dulbecco's PBS supplemented with 20% CS) for 3 min, and then equilibrated for 1 min in 15% EG, 15% DMSO, and 0.5 M sucrose in HM. Ten oocytes were loaded into each micro-pipette tip (MidAtlantic Diagnostics, Inc., Marlton, NJ, USA), and directly plunged into liquid nitrogen. Warming was performed by placing the narrow end of the micro-pipette tips directly into HM containing 0.5 M sucrose; the tips maintained in this medium for 5 min. After washing in HM, oocytes underwent an additional 3 h of maturation. They were then subjected to IVF (Day 0). After IVF, morphologically intact oocytes were cultured. Oocytes matured for 20–21 h were

used as a control. The cleavage rate at Day 3 and blastocyst rate at Day 7 to 9 were based on the number of cultured oocytes, and analyzed using the chi-square method. In experiment 1, the oocytes were vitrified with 0.5 μL of VS in micro-pipette tips with 150-, 200-, or 275-μm inner diameters (ID) (100 eggs per tip size). The number of morphologically intact oocytes was 64 (150 μm), 62 (200 μm), and 54 (275 μm). The cleavage rates of morphologically intact oocytes at Day 3 of 150 μm (45.3%) and 200-μm tips (45.2%) were significantly lower than that of 275-μm tips (53.7%) and the control (63.6%) ($P < 0.05$). The blastocyst rate of morphologically intact oocytes at Day 7 to 9 of 150-μm (9.4%) and 275-μm tips (14.8%) were significantly lower than that of the control (33.0%) ($P < 0.05$), and that of 200-μm tips (19.4%) also showed a tendency of being lower than that of the control ($P < 0.1$). In experiment 2, the oocytes were vitrified with 0.3 (70 eggs), 0.5 (60 eggs), or 1 μL (60 eggs) of VS in micro-pipette tips with 200-μm ID. The number of morphologically intact oocytes was 40 (0.3 μL), 32 (0.5 μL), and 28 (1 μL). The cleavage rates of morphologically intact oocytes at Day 3 of the 0.3 μL (45.0%), 0.5 μL (37.5%), and 1 μL solutions (35.7%) were significantly lower than that of the control (67.6%) ($P < 0.05$). However, there were no differences in the blastocyst rate of morphologically intact oocytes at Day 7 to 9 among 0.3 μL (15.6%), 0.5 μL (28.1%), and 1 μL solutions (17.9%), and control (23.9%). These results suggest that the viability of IVM bovine oocytes after vitrification may be improved by using micro-pipette tips with 200-μm ID and containing 0.5 μL of VS.

101 CRYOPRESERVATION OF RAT EPIDIDYMAL SPERM: COMPARISON OF TWO COOLING PROTOCOLS

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The present study examined post-thaw motility, plasma membrane integrity and fertility of rat epididymal sperm cooled by two procedures to +5°C, and then cryopreserved by controlled-rate freezing. Wistar rats were used in the present study. In protocol-I (2001 Reproduction 122, 463), epididymides were collected from a mature male and placed in a plastic dish containing 2 mL of freezing medium I [23% (v/v) egg yolk, 8% (w/v) lactose monohydrate and antibiotics]. The epididymides were dissected with scissors to release epididymal sperm. The semen was kept at 15°C for 30 min and then held at 5°C for 30 min. The cooling rate from 15°C to 5°C was 0.3°C min^{-1}. The cooled semen was diluted with 2 mL of freezing medium II [freezing medium I with 1.4% (v/v) Equex Stm (ES, Nova Chemical Sales, Inc., Scituate, MA, USA)]. Mixed semen was aspirated into 0.25-mL straws and exposed to liquid nitrogen (LN) vapor for 10 min. The straws were then plunged into LN. In protocol-II, epididymides were collected from a mature male and placed in 4 mL of freezing medium III [freezing medium I and 0.7% (v/v) ES]. The epididymides were dissected with scissors and held for 10 min at room temperature to release epididymal sperm. The semen was loaded into 0.25-mL straws and kept at 15°C for 15 min and then held at 5°C for 15 min. The cooling rate from 15°C to 5°C was 0.7°C min^{-1}. The cooled straws were then exposed to LN vapor for 10 min and plunged into LN. Straws were thawed in a 37°C water bath for 10 s. Thawed semen in a straw was diluted with 1 mL of KRB medium with 0.4% (w/v) bovine serum albumin (BSA, fraction V, Sigma, Tokyo, Japan) at 37°C and then incubated at 37°C in 5% CO_2 in humidified air. The percentage of motile spermatozoa was assessed visibly and determined by direct observation at 37°C under a light microscopy at 100×. The sperm membrane integrity was determined using a commercial Live/Dead sperm viability kit (Molecular Probes, Inc., Eugene, OR, USA) which differentiates between cells with intact plasma membranes and those with damaged membranes by fluorescent staining patterns observed with a fluorescence microscope (Olympus, IX-71, Tokyo, Japan). Similar levels of sperm motility were observed immediately after thawing of sperm from both protocols. However, after 2 h of incubation, the post-thaw motility of sperm frozen by protocol-II was significantly ($P < 0.01$) higher than that of protocol-I. Sperm membrane integrity immediately after thawing was also higher for sperm frozen by protocol-II (22.1% v. 9.3%, $P < 0.01$). Sperm frozen/thawed by protocol-II was inseminated into the top of the uterine horns of recipient females to confirm fertility. Two of three inseminated females became pregnant and gave birth to 5 pups. These results suggest that loading sperm into straws before cooling and subsequent slow cooling at 5°C to 0.7°C min^{-1} increases post-thaw survival of rat epididymal sperm.

102 VITRIFICATION OF IN VITRO-PRODUCED BOVINE AND OVINE EMBRYOS USING THE MINIMUM VOLUME COOLING CRYOTOP METHOD

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Considerable progress has been achieved in the cryopreservation of mammalian embryos. The use of vitrification minimizes chilling injuries by increasing cooling and warming rates. This study assesses the effect of vitrification using the minimum volume cooling (MVC) method (Kuwayama & Kato 2000 J. Assist. Reprod. Genet. 17, 477) on in vitro-produced bovine and ovine embryos. A total of 1756 ovine and 753 bovine cumulus-oocyte complexes were obtained from the abattoir and matured, fertilized (Day 0) and cultured in vitro (Walker *et al.*, 1996 Biol. Reprod. 55, 703–708, Kelly *et al.*, 1997 Theriogenology 47, 291). Overall cleavage rates were 93.7% and 80.5% respectively. Embryos were vitrified (OPS or MVC method) on Days 5 (morula, compact morula), 6 (expanded blastocyst, blastocyst, compact morula) or 7 (hatched and hatching blastocysts, expanded blastocyst, blastocyst). Embryos were equilibrated with 7.5% ethylene glycol (EG) and 7.5% dimethyl sulfoxide (DMSO) for 3 min and then exposed to 16.5% EG, 16.5% DMSO, 0.5 M sucrose and 20% FCS for 30 s. Embryos were loaded onto either an MVC plate (Cryotop, Kitazato Supply Co, Toyko, Japan) or open pulled straw (OPS) and plunged into liquid nitrogen. After 5 days, embryos were thawed directly into 1.25 M sucrose solution at 38.5°C, followed by stepwise dilution of the cryoprotectants. Embryo survival was assessed by culture to Day 8 and compared to the development of non-vitrified control embryos (Table 1). Variables were assessed using procedure CATMOD in SAS. The Cryotop method yielded a significantly higher percentage of viable ovine embryos after thawing compared with OPS ($P < 0.0001$); neither day nor treatment x day interaction was significant ($P > 0.05$). A significant interaction between vitrification treatment and day ($P < 0.007$) indicated that the percentage of hatched

embryos peaked at Day 6 using the Cryotop method compared with Day 7 for OPS. Hatching rates for fresh and vitrified embryos were similar at Day 7 and were independent of treatment. With the Cryotop method, day of vitrification did not influence the percentage of Days 6 and 7 bovine embryos that hatched after thawing but, on each day, this figure was significantly higher ($P < 0.003$ and $P < 0.0001$, respectively) than that obtained with fresh embryos. To further assess embryo viability, 36 fresh, 52 OPS and 56 Cryotop vitrified Day-6 in vitro-produced ovine embryos were transferred to synchronized recipients. Survival rates to Day 13 were 29/33 (87.9%), 23/36 (63.9%) and 42/51 (82.4%), respectively ($P < 0.05$). This study demonstrates that using the MVC Cryotop method, the viability of vitrified embryos, as assessed at Days 8 and 13, is similar to that obtained with fresh embryos.

Table 1.

	Day	Method	Embryos vitrified	Embryos surviving (%)	Embryos hatched (%)
Ovine	Day 5	Cryotop	107	107 (100.0)[a]	54 (50.5)[a]
		OPS	130	116 (89.2)[b]	69 (59.5)[ac]
	Day 6	Cryotop	220	216 (98.2)[a]	141 (65.3)[bc]
		OPS	128	114 (89.1)[b]	56 (49.1)[a]
	Day 7	Cryotop	186	181 (97.3)[a]	121 (66.9)[bc]
		OPS	119	100 (84.0)[b]	71 (71.0)[bc]
	Control	Fresh		204	145 (71.1)[b]
Bovine	Day 5	Cryotop	57	55 (96.5)[a]	18 (32.7)[a]
	Day 6	Cryotop	96	84 (87.5)[a]	44 (52.4)[b]
	Day 7	Cryotop	104	98 (94.2)[a]	66 (67.3)[c]
	Control	Fresh		104	32 (30.8)[a]

[a,b,c]Percentages within columns with a similar superscript are not significantly different ($P > 0.05$).

103 A NEW PAPER CONTAINER FOR THE VITRIFICATION OF BOVINE EMBRYOS

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Vitrification has been used to eliminate ice crystal formation during the cryopreservation of mammalian embryos. However, this method may introduce some problems such as loss of eggs during cryopreservation (EM grid) and damage to the zona pellucida. This study examined an alternative container (paper) for the vitrification of in vitro-produced bovine blastocysts. Bovine oocytes were aspirated from slaughterhouse ovaries and cultured in TCM-199 supplemented with 25 mM $NaHCO_3$, 10% (v:v) FBS, 0.22 mM sodium pyruvate, 25 mM gentamycin sulfate, 10 μg mL^{-1} FSH (Follitropin V; Vetrepharm, Canada) and 1 μg mL^{-1} estradiol-17β for 24 h. Matured oocytes were co-cultured with sperm (1–10^6 mL^{-1}) treated by percoll gradient for 42–44 h. Cleaved embryos were cultured in 50 μL CR1aa medium containing 0.4% BSA for 5 days. Blastocysts were exposed to 5.5 M ethylene glycol in CR1aa medium for 20 s. The blastocyst suspensions were vitrified by one of three methods: 1) aspiration into a 0.25-mL plastic straw (10 embryos/straw), heat sealing and immediate plunging into LN_2; 2) transfer of a (~5 μL) drop containing 10 blastocysts onto a EM grid and immediate plunging into LN_2; or 3) transfer of a (~5 μL) drop containing 10 blastocysts onto a piece of weighing paper (5 mm by 5 mm; VWR, West Chester, PA, USA) and immediate plunging into LN_2. Straws were thawed by holding in air for 10 s and then transfer into 37°C water. The embryos were recovered from the straw and transferred into a solution of 0.5 M sucrose in CR1aa at 25°C for 1 min. EM grids and paper containers were warmed by transfer into 3 mL of a solution of 0.5 M sucrose in CR1aa medium at 25°C for 1 min. Embryos were then diluted serially by transfer into 0.25 and then 0.125 M sucrose solutions (1-min steps), and then rinsed and cultured in CR1aa medium supplemented with 10% FBS. After thawing, the recovery rates of embryos from EM grids, straws and paper containers were not significantly different (Table 1). Broken zonae pellucidae were observed after thawing of embryos recovered from straws and EM grids, but not from the paper container. The survival rates of blastocysts cryopreserved on EM grids and paper containers (respectively, 78.1 and 77.1%) were significantly higher ($P < 0.05$) than that of straws (52.1%). The in vivo developmental potential of blastocysts vitrified on EM grids and paper containers was assessed by the transfer of, respectively, 102 and 3 thawed embryos into recipient cows. Pregnancy rates were, as anticipated, 28 and 67%. These results suggest that paper may be an inexpensive and useful container for the cryopreservation of mammalian embryos.

Table 1. The viability of vitrified-thawed bovine embryos using various containers

Container type	No. of embryos	No. (%) of embryos recovered	No. (%) of embryos lost	No. (%) of zonae broken	No. (%) of embryos surviving
EM grid	735	622 (84.6)	89 (12.1)	32 (4.4)	486 (78.1)[a]
Straw	137	121 (88.3)	16 (11.7)	8 (5.8)	63 (52.1)[b]
Paper	79	74 (93.7)	7 (8.9)	0 (0.0)	57 (77.1)[a]

[a,b]Different superscripts within columns denote significant differences ($P < 0.05$).

104 EFFECT OF HATCHING STATUS ON VITRIFICATION OF CLONED BOVINE BLASTOCYSTS

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Bovine blastocysts produced by nuclear transplantation have mechanical slits in their zonae pellucidae, and therefore initiate hatching earlier than the non-manipulated embryos. The present study was undertaken to examine whether the hatching stage of cloned blastocysts is among the factors influencing their survival after vitrification and warming. Cloned bovine blastocysts were produced by using adult ear fibroblast cells as reported previously (Parnpai *et al.*, 2002, Theriogenology 57, 443), except that fused couplets were co-cultured with bovine oviductal epithelial cells in mSOFaa medium supplemented with 0.1% linoleic acid-albumin (LAA) + 0.2% BSA (Hochi *et al.*, 1999, Theriogenology 52, 497–504). Hatching blastocysts harvested on Day 7 were classified into one of three groups according to the ratio of extruding embryonic diameter from zona (D2) to embryonic diameter inside the zona (D1); category-A: D2/D1 = 0.01–0.70; category-B: D2/D1 = 0.71–1.00; category-C: D2/D1 = 1.01–1.70. The blastocysts were first exposed to 10% DMSO + 10% ethylene glycol in TCM199 + 20% FCS for 2 min, and then equilibrated in 20% DMSO + 20% ethylene glycol + 0.5 M sucrose with or without 10% Ficoll in TCM199 + 20% FCS for 30 s. One to three blastocysts were placed on a Cryotop sheet (Kitazato Supply Co., Tokyo, Japan) and vitrified in liquid nitrogen. The samples were warmed in 0.5 M sucrose solution for 2 min and transferred into TCM199 + 20% FCS in five steps (5 min per step). The post-warm survival of the blastocysts was assessed by in vitro culture for 24 h. When Ficoll-free vitrification solution was used, post-warm survival rate of the category-A blastocysts (77%, 23/30) was not significantly different (ANOVA test) from those of category-B and category-C blastocysts (74%, 20/27; and 80%, 24/30; respectively). Inclusion of 10% Ficoll in the vitrification solution did not improve (ANOVA test) the post-warm survival rates of cloned blastocysts (category-A: 65%, 22/34; category-B: 54%, 15/28; category-C: 59%, 19/32). Groups of fresh nonsurgical embryos, vitrified with or without Ficoll, yielded 66.7% (4/6), 66.7% (2/3) and 40.0% (2/5), respectively, of recipients pregnant at 48 days of gestation. In conclusion, cloned bovine blastocysts, regardless of their hatching stages, were relatively resistant to cryopreservation by vitrification. (Supported by Thailand Research Fund and R&D Fund of Suranaree University of Technology.)

105 VITRIFICATION OF BOVINE EMBRYOS USING THE CLV METHOD

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Vitrification has become the preferred method for cryopreserving in vitro-produced bovine embryos (IVP). Here we introduce a technique for vitrification developed at CryoLogic (the CLV Method), in conjunction with a study comparing the post-thaw viability of IVP embryos frozen by the widely used open pulled straw method (OPS—Vajta *et al.*, 1997 Cryo-Letters 18, 191) and the new CLV Method. Vitrification on thin metal surfaces has been explored and demonstrated previously (Le Gal & Massip 1999, Cryobiology 38, 290), and Dinnyes presented a Solid Surface Vitrification (SSV) (Dinnyes *et al.*, 2000, Biol. Reprod. 63, 513). The CLV Method utilizes vitrification on the surface of a solid metal block. This surface has been custom shaped and treated to enhance vitreous formation. The method also includes handling, storage and thawing protocols designed to avoid damage from crystallization of the unstable glass. Briefly, the block is precooled in LN2 to −196°C. Up to 10 embryos are collected into a droplet of medium (3 μL), on the end of a fibre carrier attached to a handle. The droplet is presented to the vitrification surface, where it is converted into a glass bead by cooling rates comparable to that of plunging into solid/liquid phase nitrogen (−210°C) (Arav *et al.*, 2001 Theriogenology 55, 313). The glass bead, fibre and handle are transferred quickly into a half-sealed, precooled straw, and the handle seals the open end. A bead is thawed very rapidly by removing the handle, fibre and bead from the straw and transferring the bead into washing medium. COCs collected from bovine ovaries obtained from abattoirs were matured, fertilized and cultured for 6 days (Fry *et al.*, 2003 Theriogenology 59, 446). Embryos reaching the blastocyst or expanding blastocyst stage of development were graded (Grades 1, 2, and 3), equilibrated for 5 min in HEPES-199 medium with 20% FCS (HFm), placed in HFm with 10% EG, and 10% DMSO (VS1) for 2 min, and then transferred to HFm with 20% EG, 20% DMSO (VS2) for 30 s (Vajta). Between 5 and 10 IVP embryos were processed and collected for vitrification, either in an OPS plunged into LN2, or in a 3 μL droplet vitrified by the CLV Method. The two sets of specimens were stored in LN2, and later thawed. Both OPS tips and beads were thawed in 0.5 mL of HFm with 0.2 M sucrose at 39°C. Embryos were maintained at 39°C, examined after 5 min for contraction, and again after 6 h for re-expansion. They were then transferred to culture medium, incubated and examined at 24 and 48 h to assess hatching. As shown in Table 1, the CLV method appears to be satisfactory for maintaining membrane integrity (expansion) and developmental potential (hatching) for even poorer grade embryos, that might be more sensitive to the stresses of cryopreservation.

Table 1. Re-expansion and hatching rates of graded thawed bovine embryos vitrified by OPS or CLV methods

Embryo Grade	Method	# Frozen/thawed	# Re-expanded (%)	# Hatched (%)
Grade 1	OPS	92	81 (88)	75 (82)
	CLV	78	72 (92)	69 (88)
Grade 2/3	OPS	53	35 (66)	24 (45)
	CLV	35	31 (91)	26 (76)

No difference in re-expansion or hatching of Grade 1 embryos by either method. More ($P < 0.05$) Grade 2 and 3 embryos re-expanded and hatched after freeze thawing using CLV than OPS.

106 EVALUATION OF ADDITION OF REDUCED GLUTATHIONE TO COOLING MEDIUM ON IN VITRO FERTILITY AND ACROSOME REACTION IN BOAR SPERMATOZOA

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The process of cooling to 5°C prior to freezing produces physical and chemical stress on the sperm membrane associated with oxidative stress and reactive oxygen species (ROS) generation that reduces sperm viability and fertilizing ability. The addition of antioxidants to cooling medium could prevent the formation of ROS and improve the seminal parameters. The aim of these experiments was to investigate the effects of addition of reduced glutathione (GSH) to cooling extenders on (1) plasma membrane integrity, (2) acrosome reaction induction by ionophore A 23187 or progesterone, and (3) in vitro fertilization. Ejaculate-rich fractions from three mature pietrain boars were diluted in Beltsville Thaw Solution (BTS) extender and cooled to 15°C over 2 h (group C). Thereafter, sperm were centrifuged and diluted in lactose/egg-yolk extender with 0 mM (group 0), 1 mM (group 1) or 5 mM (group 5) of GSH, cooled to 5°C over 2 h. The acrosome reaction was then induced by 1 μM calcium ionophore or 10 μM progesterone in TALP medium and incubated in 5% CO2, 38.5°C for 30 or 45 min, respectively. Membrane integrity was evaluated by propidium iodide, and acrosomal status was monitored by means of FITC-labeled peanut agglutinin. Finally, in vitro fertilization was performed with these four spermatozoa groups as described previously (Matás *et al*. 2003 Reproduction 125, 133–141). ANOVA analysis revealed that the addition of GSH had no effect on the membrane integrity (ranged 58.8 to 66.9) or acrosome reaction induction (ranged 24.3 to 28.2, and 55.7 to 41.4 for progesterone and calcium ionophore, respectively). However, the results of the penetration assay revealed that the cooling affected the penetration rate and the number of sperm per oocyte (Table 1), and this assay is better than the others to predict changes in the spermatozoa functionality (Gadea J and Matás C 2000 Theriogenology 54, 1343–1357). In conclusion, the cooling process affects the in vitro fertilization, but the addition of GSH to the medium did not influence the parameters studied. Supported by AGL2000-0485-CO2-01.

Table 1. Homologous in vitro penetration

	n	Penetration rate (%)	Sperm per penetrated oocyte
Group C	283	62.19 ± 2.89^{a}	4.19 ± 0.34^{a}
Group 0	269	44.98 ± 3.04^{b}	2.39 ± 0.21^{b}
Group 1	270	47.78 ± 3.05^{b}	2.19 ± 0.21^{b}
Group 5	270	45.93 ± 3.04^{b}	2.42 ± 0.20^{b}

[a,b]Indices without common superscripts differ, $P < 0.001$

107 TOXICITY OF ETHYLENE GLYCOL ON FROZEN AND THAWED IVP EMBRYOS IN DIRECT TRANSFER METHOD

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Ethylene glycol (EG) is a cryoprotectant which is highly permeable to mammalian embryos. But the toxicity of this cryoprotectant for embryos after thawing has not been investigated. The aim of this study was to determine the toxicity of EG to embryos frozen and thawed by a direct transfer method. In vitro-produced Day 7 blastocysts ($n = 529$) of grade 1 quality were used in this study. Embryos were frozen in 1.5 M EG in Dulbecco's PBS (DPBS) supplemented with 0.1 M sucrose, 4 mg mL^{-1} BSA and 20% fetal calf serum (FCS). Embryos were transferred into freezing medium, loaded into 0.25-mL straws and kept for more than 15 min for equilibration; then the straws were plunged into a −7°C methanol bath of a programmable freezer for 1 min, seeded at −7°C, held at −7°C for 14 min, cooled to −30°C at the rate of -0.3°C min^{-1} and then plunged into liquid nitrogen. The straws were thawed by holding in air for 6 sec, and then placed in water at 30°C for 15 s. After thawing, the straws were held for 0, 10, 20, 30 and 60 min (holding time) at either 38.5 or 26.0°C. Ethylene glycol was removed from the embryos by placing them into DPBS supplemented with 20% CS at 38.5°C more than 20 min. The embryos were cultured in TCM-199 supplemented with 20% FCS and 0.1 mM β-mercaptoethanol under a gas phase of 5% CO_2 in air at 38.5°C for 72 h. Viability of embryos was evaluated at 0-, 24-, 48- and 72-h incubation by their morphological development. Data were analyzed by ANOVA. There was no significantly difference in the survival rate of thawed embryos held at 38.5°C or 26.0°C for the same holding periods. The survival rate of the thawed embryos held at 38.5°C decreased significantly when the holding period exceeded 30 min compared with no holding period after 24- and 72-h culture ($P < 0.05$, respectively). On the other hand, the survival rate of the thawed embryos held at 26.0°C decreased significantly when the holding time was 60 min compared with less than 20 min of holding after 24-h culture, and less than 10 min after 72-h culture ($P < 0.05$, respectively). Therefore, toxicity of EG was observed when thawed embryos were held for 30 and 60 min at 38.5°C and 60 min at 26.0°C. These results suggest the toxicity of EG in direct transfer methods can be avoided by transferring the embryos within 20 min after thawing.

Table 1.

Temperature (°C)	Holding time (min)	No. of embryos	Survival rate (Mean ± SEM) (%)	
			24 h	72 h
38.5 ($n = 7^x$)	0	51	71.1 ± 25.7^{a}	71.1 ± 25.7^{a}
	10	52	54.0 ± 16.1^{ab}	47.6 ± 16.6^{ab}
	20	51	61.7 ± 17.4^{ab}	59.9 ± 16.4^{ab}
	30	51	40.7 ± 29.6^{b}	40.7 ± 29.6^{b}
	60	49	44.0 ± 23.2^{b}	41.8 ± 21.9^{b}
26.0 ($n = 9^x$)	0	54	56.9 ± 28.5^{ab}	51.9 ± 28.8^{a}
	10	55	77.5 ± 17.5^{a}	64.2 ± 20.7^{a}
	20	58	58.7 ± 23.4^{ab}	50.3 ± 30.5^{ab}
	30	53	53.3 ± 19.1^{bc}	50.8 ± 15.9^{ab}
	60	55	32.2 ± 27.5^{c}	28.4 ± 23.9^{b}

[x]Number of replicates. Values in same columns of each temperature with different superscripts differ ($P < 0.05$).

108 CRYOPRESERVATION OF GERMPLASM FROM HERITAGE BREEDS OF DOMESTIC LIVESTOCK: SEASONAL EFFECTS ON SUPEROVULATION RESPONSE AND EMBRYO PRODUCTION

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There is an urgent need to preserve the genetic diversity of rare breeds of domestic livestock. However, it is unknown if conventional superovulation methods are effective in unimproved breeds. The establishment of germplasm cryostorage banks requires the ability to produce viable preimplantation stage embryos. To our knowledge, we describe here the first successful production and cryopreservation of embryos from Gulf Coast Native (GCN) sheep and Tennessee Myotonic (TM) goats. Mature GCN ewes and TM does were synchronized (prostaglandin F2α, 7.5–10 mg i.m.) and all TM does, and some GCN ewes, were treated with progesterone (20 mg sq, 5× on alternate days, prior to FSH treatment, then 10 mg i.m. on second day of FSH treatment). Animals were superovulated (FSH, 50–40–30 mg bid i.m., decreasing over 3 days) both in April (cycling) and June (anestrous). Females were bred by natural service with a proven male over 24 hr, and embryos were collected surgically from the uterus on Day 7. FSH-stimulated cycling TM does produced a total of 15.3 ± 3.0 CL and 10.7 ± 7.2 morulae/animal, whereas FSH-treated anestrous TM does yielded 9.0 ± 2.8 CL and 7.0 ± 8.4 embryos per animal. Recovered goat embryos were morphologically of good-excellent quality, and were cryopreserved using a conventional multi-step freezing protocol. Cycling GCN ewes responded to FSH simulation and produced 9.7 ± 2.5 CL per animal, whereas the ovaries of non-cycling ewes did not respond to exogenous FSH treatment (0 CL). This report documents that TM does will effectively respond to FSH superovulation treatment and produce viable embryos during the breeding season and during the anestrous period. In contrast, GCN ewes appear to respond only to exogenous FSH during the breeding season. Collectively, these results demonstrate that the TM and GCN landrace breeds of goats and sheep display differential responses to hormone manipulation; yet, with breed-optimized protocols, preimplantation stage embryos can be obtained for long-term cryostorage of germplasm of heritage breeds of livestock. Supported by and in partnership with SVF Foundation.

109 REFREEZING STALLION SPERMATOZOA FOR ASSISTED REPRODUCTION

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Equine semen is commonly frozen in 0.5-mL straws at a concentration of 400 million spermatozoa per mL. Equine pregnancies have been produced in the past few years using assisted reproduction techniques that require very few sperm, such as sperm injection and gamete intrafallopian transfer. Consequently, thawing even a single straw would waste a high percentage of the sperm. The goals of this study were to: (1) evaluate the motility and viability of cryopreserved spermatozoa that had been thawed and refrozen, and (2) compare the effect of serial dilution on refreezing parameters. It was hypothesized that both motility and viability would be significantly decreased as a consequence of refreezing, but sufficient live, motile spermatozoa would be present for assisted reproductive techniques. Semen from six stallions of mixed light horse breeds was collected, evaluated, and frozen in lactose-EDTA in 0.5-mL straws. Semen from each stallion was subsequently thawed, held at room temperature for 10 minutes, and then refrozen in 0.25-mL straws. Additional semen from the initial freeze was thawed, diluted in the original extender, and refrozen at concentrations of 40×10^6, 4×10^6, 4×10^5, and 4×10^4 spermatozoa per mL. Refrozen semen was evaluated for motility visually and by CASA. Viability of refrozen semen was determined by flow cytometry following staining with propidium iodide (PI). Percentages of motile spermatozoa prior to freezing, after the first freezing, and after the second freezing were compared using the PROC GLM model in SAS. All values are presented as the mean ± standard deviation. Total motility decreased sigificantly ($P < 0.05$) from $91.8 \pm 3.1\%$ prior to freezing to $64.2 \pm 7.7\%$ after the first freezing and $45.7 \pm 10.4\%$ after the second freezing. However, only a tendency ($P = 0.09$) toward a decrease in sperm viability following refreezing as measured

by PI staining was noted. Dilution of sperm prior to refreezing did not significantly effect ($P > 0.05$) subsequent motility or viability parameters. This study demonstrated that refrozen equine semen retained approximately 70% of the initial post-thaw motility. Dilution prior to refreezing would allow for the reallocation of a single 0.5-mL straw into hundreds or thousands of smaller straws for future use in assisted reproduction. Refreezing would allow for the judicious use of valuable stallion semen in limited supply.

110 OSMOTIC STRESS ON THE CELLULAR ACTIN FILAMENT ORGANIZATION OF IN VITRO PRODUCED PORCINE EMBRYOS

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Disruption of the actin cytoskeleton is one of the leading causes in low survival of pig embryos after cryopreservation (Dobrinsky *et al.*, 2000 Biol. Reprod. 62, 564–570). In this study, the effect of osmotic stress on cellular actin filament organization in porcine embryos produced in vitro was studied. Excellent quality Day 6 (fertilization = Day 0) porcine blastocysts were randomly exposed to 6 different anisosmotic sucrose solutions (75, 150, 210, 600, 1200, 2400 mOsm) for 10 min. Embryos were then returned to embryo culture medium (NCSU-23) after washing with NCSU-23, and cultured under 38.5°C, 5% CO_2 in air with maximal humidity for them to recover. Blastocysts cultured in NCSU-23 medium (280 mOsm) served as a control for embryos with intact actin filament organization. Blastocysts treated with 7.5 $\mu g\,mL^{-1}$ cytochalasin-b for 60 min served as a control for embryos with F-actin depolymerization. Eighteen hours post-anisosmotic treatments, all blastoysts were fixed in 3.7% paraformaldehyde in PBS for 60 min and stored in PBS with 0.1% Triton X-100 and 0.2% sodium azide at 4°C. Staining of actin filaments was performed according to procedures described earlier (Wang *et al.*, 1999 Biol. Reprod. 60, 1020–1028). Embryos were blocked in PBS with 20 $mg\,mL^{-1}$ BSA and 150 mM glycine for 30 min. After being washed in PBS with 0.1% Tween 20 for 60 min, embryos were stained with 10 $U\,mL^{-1}$ Alexa Fluor 488 phalloidin in PBS with 0.1% Tween 20 at 38.5°C for 60 min, and then washed twice in PBS with 0.1% Tween 20 for 60 min each. The status of actin filaments in embryonic cells was examined by confocal microscopy. Integrity of cellular actin filaments was classified as either intact or disrupted according to the distribution within embryonic cells. Blastocysts were then classified according to the status of actin filaments in embryonic cells. Data were analyzed using logistic regression. Results from 7 replicates are displayed in Table 1. There was a significant relationship between osmotic treatment levels and the probability of blastocysts with disrupted cellular actin filaments ($P < 0.0001$). These data support the hypothesis that porcine embryos are very sensitive to osmotic changes. Ongoing experiments will assess the extent of actin disruption required to significantly reduce developmental competence of pig blastocysts. This study was supported by Monsanto Company.

Table 1. Cellular actin filament integrity of in vitro produced porcine blastocysts after being treated with sucrose solutions with different osmolalities (mOsm)

Osmolality	75	150	210	280	600	1200	2400
Intact/total*	20/45	24/43	26/43	35/41	34/44	21/45	18/44
Percentage	44	56	60	85	77	47	41

*Number of blastocysts with intact cellular actin filament organization/total blastocysts examined.

111 VOLUMETRIC CHANGES OF BOVINE OOCYTES RESULTING FROM DEHYDRATION IN DISACCHARIDE SOLUTIONS

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Vitrification of mammalian oocytes employs hypertonic solutions of cryoprotectant additives (CPAs), causing a rapid loss of water from the cell. This severe dehydration can result in osmotic shock. Oocytes are known to be altered by abrupt changes in osmotic pressure (1987 Cryobiology 24, 387–402), which may result in ultrastructural changes. Therefore, the objective of this study was to examine the effects of severe dehydration of mature bovine oocytes on the cytoskeletal arrangement of microtubules and actin filaments as well as the metaphase spindle prior to vitrification. In Experiment I, oocytes ($n = 30$) obtained from Ovagenix (San Angelo, TX, USA) were sequentially exposed (5 or 6/treatment group) to increasing hypertonic solutions of two saccharides (0.15, 0.3, 0.5, 0.65, 0.99 M sucrose and 0.125, 1.25, 0.35, 0.55, 0.65 M trehalose) prepared in TCM-199 or M2 medium. Control oocytes ($n = 22$) were subjected to isotonic control solutions (TCM-199 and M2 alone). Relative average volumes of oocytes were determined as previously described and then Boyle vant Hoff plots were constructed. In this study, bovine oocytes behaved similarly when exposed to increasing osmolalities of sucrose and trehalose in both media. The oocytes exhibited a linear decrease in relative volume as a function of 1/(osmotic pressure)to a minimum of approximately 32% of the isotonic volume for 0.99 M sucrose in M2 medium ($r^2 = 0.9785$) (Graphpad 2.04, GraphPad Software, San Diego, CA, USA). In the second experiment, partially denuded bovine oocytes ($n = 106$) were sequentially dehydrated to determine the effect of dehydration on cytoskeletal structures and the meiotic spindle. Oocytes were suspended in solutions of 0.99 M sucrose or 0.65 M trehalose in either TCM-199 or M2 medium (concentration of sucrose and trehalose where maximum dehydration occurred in the first

experiment). Correspondingly, similar oocytes ($n = 30$) were allotted to either TCM-199 or M2 medium control groups. Oocytes were subsequently fixed, immunolabelled for tubulin (rat anti-α-yeast tubulin and anti-rat FITC) and actin (rhodamine phalloidin), stained for DNA (Hoechst 33342), and then mounted for analysis. Cytoskeletal arrangement and DNA localization were visualized using fluorescence and laser-scanning microscopy. Microtubules in control oocytes were intimately associated with the meiotic spindle, which appeared as a symmetrical, barrel-shaped structure with anastral poles. Intact spindles were observed in 63% of the control oocytes compared with 0% in all treatment groups, with the exception of 3% of the oocytes suspended in 0.99 M sucrose in TCM-199. Co-localization of actin with tubulin was observed in all oocytes with intact spindles. In conclusion, bovine oocytes respond as perfect osmometers when suspended in hypertonic solutions of saccharides. Oocytes exposed to such solutions exhibited extensive disruption of their meiotic spindles, which would undoubtedly result in reduced fertilization and abnormal development. Further study is needed to determine whether the effects of dehydration can be reversed by sequential rehydration.

112 EFFECT OF THE IVM PROTOCOL OF BOVINE OOCYTES ON SURVIVAL RATES AFTER VITRIFICATION BY OPEN PULLED STRAW METHOD

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The meiotic stage and the cryopreservation protocol influence the ability of the oocytes to survive cryopreservation. The in vitro maturation (IVM) methods affect nuclear and cytoplasmic maturation and, consequently, the developmental competence of the oocytes. On the other hand, the cytoplasm of the bovine oocyte contains large amounts of lipids which, as demonstrated in the bovine embryo (Díez *et al*., 2001 Theriogenology 55; 923–936), can negatively affect post-thaw survival. The aim of this work was to analyze the effect of fetal calf serum (FCS) during IVM on the freezability of the bovine metaphase II oocyte. Cumulus-oocyte complexes (COCs) were recovered from slaughterhouse ovaries. Oocytes with compact cumulus cells and evenly granulated cytoplasm were matured for 22 h in TCM199, $NaHCO_3$, FSH, LH and 17βestradiol. Approximately half of the oocytes were allowed to mature in 10% FCS, and the remainder were matured in polyvinyl-alcohol (PVA; 0.3 g L^{-1}). For vitrification, oocytes were matured for 22 h, partially denuded of cumulus cells, and then vitrified (v-FCS and v-PVA) by the OPS system (Vajta *et al*. 1988 Mol. Reprod. Dev. 51; 53–58). Fresh untreated controls (c-FCS and c-PVA) were allowed to mature for 24 h and immediately fertilized in modified TALP medium with swim-up separated sperm, and cultured. After warming and dilution, vitrified oocytes were cultured in IVM medium for 2 h and then fertilized (Day 0). Presumptive zygotes with normal morphology were cultured in SOFaa + amino-acids + myo-inositol + 5% FCS (Day 3), and oocytes with a degenerated appearance were counted and discarded. Data were analyzed by ANOVA and Duncan's test. Results are shown in the Table 1. After warming, we observed severe cryodamage in both v-FCS and v-PVA groups. Rates of degenerated oocytes were 17.8 ± 9.6 and 12.0 ± 9.6 for v-FCS and v-PVA groups, respectively ($P > 0.05$). The presence of PVA instead of FCS did not improve the blastocyst rates obtained from vitrified/warmed oocytes. The use of PVA during IVM (c-PVA) yielded lower ($P < 0.05$) blastocyst rates compared to the FCS control (c-FCS). Ultrastructural studies are in progress to analyze alterations in meiotic spindle, cytoplasmic organelles and cortical granules as possible causes of reduced oocyte competence after vitrification. Supported by CICYT, AGL2001-379.

Table 1.

IVM	N	% Cleaved Day 3	% 5–8 cells Day 3	% M + Bl[d] Day 6	% Blastocysts Day 7	Day 8
v-FCS	167	51.6 ± 6.4^{a}	21.4 ± 5.2^{a}	6.8 ± 5.3^{a}	1.3 ± 3.6^{a}	1.8 ± 3.6^{a}
v-PVA	176	47.8 ± 6.4^{a}	22.4 ± 5.2^{a}	4.6 ± 5.3^{a}	0.0^{a}	0.4 ± 3.6^{a}
c-FCS	247	79.3 ± 5.8^{b}	63.4 ± 4.7^{b}	41.4 ± 4.8^{b}	46.6 ± 4.1^{b}	46.6 ± 4.1^{b}
c-PVA	217	82.3 ± 5.8^{b}	53.5 ± 4.7^{b}	28.7 ± 4.8^{b}	23.7 ± 4.1^{c}	25.3 ± 4.1^{c}

N: number of zygotes cultured (6 replicates). [d]Morulae + Blastocysts rate; development rates refer to zygotes cultured. Data are LSM ± SE. Superscripts express significant differences: [a,b,c] ($P < 0.05$)

113 COMPARISON OF TWO ETHYLENE GLYCOL EQUILIBRATION TREATMENTS FOR THE QUICK FREEZING OF IN VITRO-PRODUCED BOVINE EMBRYOS

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The aim of this study was to compare two ethylene glycol (EG) equilibration procedures for the quick freezing of in vitro-produced bovine embryos. Cumulus-oocyte complexes (COCs) were collected from slaughterhouse ovaries. COCs were matured in TCM199 containing 10% of bovine fetal serum, LH, FSH and E2, and fertilized. Presumptive zygotes were co-cultured in TCM199 with a granulosa cell monolayer, at 39°C in humidified atmosphere of 5% CO_2 in air. Grade 1, expanded blastocysts ($n = 761$) were selected 7 and 9 days after insemination and randomly distributed to one of eight treatment groups. In Equilibration Procedure 1, embryos were exposed to 10% EG for 5 min, and then to 17%, 22% or 28% EG for

60 s (respectively referred to as EG 17, EG 22 and EG 28). In Equilibration Procedure 2, embryos were exposed to the same EG solutions as in Equilibration Procedure 1, but the period of exposure was 10 min to 10% EG and 30 s to EG 17, EG 22 and EG 28. In Equilibration Procedure 3 (slow-freezing controls), embryos were exposed to 10% EG for either 5 or 10 min and then cryopreserved by slow-freezing method at 1.2°C/min. In all treatment groups, EG solutions were prepared in PBS + 0.2% BSA, and embryos were exposed to EG solutions at 22°C. Embryos were loaded into 0.25 mL straws and heat-sealed. Straws were cooled in liquid nitrogen vapor for 2 min, and then plunged into and stored in liquid nitrogen. Straws were thawed in room temperature air for 10 s, and then in 25°C water for 20 s. Thawed embryos were diluted by transferring them into 0.5 ml of PBS + 0.2% BSA + 0.3 M sucrose for 3 min, and then 0.5 mL of PBS + 0.2% BSA for 3 min. Embryos were co-cultured on granulosa cell monolayer in TCM199 and evaluated after 24 h for blastocyst re-expansion (EXP), and again at 48, 72 and 96 h for hatching (HAT). A total of 724 in vitro-produced bovine blastocysts were used as controls to determine hatching rates. The results are presented in the Table. Embryos exposed to 10% EG for 10 min (Equilibration Procedure 1) yielded significantly higher rates of blastocyst re-expansion and hatching when compared to embryos exposed for 5 min (Equilibration Procedure 2, $P < 0.05$). These results suggest that quick freezing of in vitro-derived bovine embryos may be an alternative to vitrification; however, additional studies are needed to optimize cryopreservation protocols and increase post-thaw survival. This project was supported by FAPESP (01/11266-4)

Table 1. Effect of equilibration procedure on in vitro re-expansion and hatching rates of embryos cryopreserved by slow and quick freezing methods

	EG 10		EG 17		EG 22		EG 28		Control HAT
	EXP	HAT	EXP	HAT	EXP	HAT	EXP	HAT	
5 min	54.3* (92)^{aA}	30.4 (92)^{aA}	14.1 (106) ^{bA}	0.9 (106)^{bA}	10.3 (97)^{bA}	1.0 (97)^{bA}	32.3 (93)^{cA}	14.0 (93)^{cA}	70.2 (724)^{dA}
10 min	77.9 (104)^{aB}	57.7 (104)^{aB}	15.4 (91)^{b}	2.2 (91)^{b}	24.2 (95)^{bB}	12.7 (95)^{cB}	21.7 (83)^{b}	7.2 (83)^{bc}	70.2^{d}

Values with different superscripts (^{A,B}) within columns differ, $P < 0.05$. Values with different superscripts (^{a,b,c,d}) within rows for EXP or HAT differ, $P < 0.05$, chi-square analysis. *Percent of embryos; () = number of embryos. EG 10 = slow freezing, 1.8°C/min.

114 EFFECT OF CYTOCHALASIN B TREATMENT ON VITRIFICATION OF CAPRINE PARTHENOGENIC BLASTOCYSTS

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Production of human recombinant proteins in the milk of transgenic animals has been shown to be a viable production system. Protection of the animal genetics involved is paramount. Vitrification of embryos is a simple, time-efficient way of preserving an animal's genetics without the formation of damaging ice crystals during the freezing process. Cytochalasin B has been shown to increase the viability of porcine blastocysts by reducing damage to microfilaments and other cytoskeletal components. These experiments utilized caprine parthenogenic blastocysts as a model to compare the viability of parthenotes treated with or without cytochalasin B prior to and during vitrification. Abattoir oocytes were in vitro-matured in M199 with 10% goat serum containing FSH, LH and gentamycin for 18 to 21 h. Parthenogenic blastocysts were produced by treating in vitro matured abattoir oocytes with ionomycin for 5 min (5 μM) and with 6-dMAP (3 mM) for 3 h followed by culturing in SOF + 0.8% BSA for 7 to 8 days at 38°C with 6% O_2, 5% CO_2, and 89% N_2 in a modular incubator chamber. The experimental group was treated with cytochalasin B (5 μg/mL)in the culture media for 30 to 45 min prior to and thereafter throughout the vitrification process. All blastocysts (both the experimental group and the control group) were washed through two ovum culture media (OCM) droplets for 5 min each. The blastocysts were incubated in vitrification solutions 1 and 2 (10% glycerol in OCM and 10% glycerol + 20% ethylene glycol in OCM, respectively) for 5 min each, followed by vitrification solution 3 (25% glycerol + 25% ethylene glycol in OCM). They were then aspirated immediately into a 0.25 cc cryopreservation straw, followed by an air bubble, and then a 0.25 M sucrose solution in OCM. The straws were immediately plunged into liquid nitrogen and stored at −196°C. One to four days later, straws were thawed in air for 5 s at room temperature, then in 22°C water for 15 s. After thawing, the contents of the straw were expelled, mixed, held for 5 min, and finally placed in OCM for 5 min. Recovered embryos were placed in SOF + 20% FBS and incubated at 38°C with 5% CO_2 in air overnight. Viability was determined by re-expanding and subsequent hatching of the blastocyst. As shown in Table 1, there were no significant differences between re-expansion and hatching of blastocysts with cytochalasin B treatment compared to blastocysts not treated with cytochalasin B. These results suggest that, unlike porcine embryos (Dobrinsky *et al*., 2000 Biol Reprod 62, 564–570), cytochalasin B treatment does not improve the post-thaw viability of vitrified caprine parthenogenic blastocysts.

Table 1.

	# Experiments	# Vitrified	# Recovered post thaw (%)	# Re-expanded (%)	# Hatched (%)
Cytochalasin β	26	76	73 (96)	16^{a} (22)	9^{a} (12)
No Cytochalasin β	25	40	36 (90)	7^{a} (19)	4^{a} (11)

%'s based on # recovered post thaw. Data were analyzed by Chi-square test. ^{a,b} within columns differ significantly. $P < 0.05$.

115 DIMETHYLSULPHOXIDE AND 1,2-PROPANEDIOL DIFFERENTIALLY CRYOPROTECT BOVINE AND PORCINE PRIMORDIAL, PRIMARY AND SECONDARY FOLLICLES

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Ovarian tissue cryopreservation is of interest for many areas of assisted reproduction. Normal structure and intra- as well as intercellular organization have to be maintained in order to preserve follicle viability. In the present work we studied the effects of two largely used cryoprotectants, namely, dimethylsulphoxide (DMSO) and 1,2-propanediol (PROH), on follicle morphology. Experiments were carried out on bovine and porcine ovaries and the two cryoprotectants were assessed for their ability to preserve structural integrity of primordial, primary and secondary follicles, in order to investigate the protective effects of these molecules on specific developmental stages. Fragments from each ovary were divided in three groups: a) immediately fixed (control); b) cryopreserved in 1.5 M DMSO; c) cryopreserved in 1.5 M PROH. To allow equilibration with the cryoprotectant, samples were held for 30 min at 4°C in order to minimize toxic effects. Cryopreservation was carried out in a controlled rate freezer (Planer Planerple, Sunbury, Middlesex, UK), with the following protocol: precooling (4°C); cooling at −2°C/min to 9°C; seeding at 9°C and standby for 10 minutes; cooling at −0.3°C/min to 40°C and 10°C/min to 140°C; plunging into liquid nitrogen. Samples were rapidly thawed. Dilution of cryoprotectants was carried out in 3 steps of ten minutes each at 4°C (1 M; 0.5 M; 0 M). Samples were then fixed, paraffin embedded, serially sectioned and evaluated with a Nikon TE200 inverted microscope. Follicles of each developemental stage were scored in three categories using the criteria previously described by Paynter *et al*. (1999, Cryobiology 38, 301–309) and presented in the table below as I = Intact; SA = Small Abnormalities; GA = Great Abnormalities. Statistical differences were assessed by the chi-square test ($P < 0.05$). The results obtained showed that both cryoprotectants are effective for the preservation of secondary follicles. The use of PROH was unable to protect primary and primordial follicles in both species. Conversely, DMSO showed a satisfying cryoprotecting effect for pig primary and bovine primordial follicles but had a poor protecting capability for pig primordial and bovine primary follicles. Altogether the present results suggest that the choice of the cryoprotectant needs to be carefully targeted in relation to the follicular stage and the species of interest. This work was funded by Industria Farmaceutica Serono SpA.

Table 1.

	Primordial			Primary			Secondary		
	%I	%SA	%GA	%I	%SA	%GA	%I	%SA	%GA
Pig		$n = 341$			$n = 159$			$n = 54$	
Control	87.6	11.3	1.1	81.4	9.3	9.3	88.9	0	11.1
PROH	67.7*	11.5*	20.8*	50*	33.3*	16.7*	67.6	29.4	3
DMSO	78.7*	9.8*	11.5*	84.6	15.4	0	41.7	33.3	25
Bovine		$n = 62$			$n = 110$			$n = 47$	
Control	87.5	12.5	0	73.5	20.6	5.9	75	25	0
PROH	20*	55*	25*	36.7*42	50*	13.3*	42.1	42.1	15.8
DMSO	61.5	23	15.5	28.3*	47.8*	23.9*	58.3	16.7	25

Results are expressed as %; * indicates values different from control within the same species ($P < 0.05$).

116 DEVELOPMENT INTO BLASTOCYSTS OF SWAMP BUFFALO OOCYTES AFTER VITRIFICATION AND NUCLEAR TRANSFER

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Oocyte cryopreservation in the domestic species is still at the experimental stage, but recent studies indicated that vitrification characterized by ultra-rapid cooling rate is promising for cryopreservation of bovine oocytes. In the present study, denuded buffalo oocytes were vitrified by minimum volume cooling procedure (Kuwayama and Kato, 2000, J Assist Reprod Genet 17, 477) after IVM or after IVM and enucleation, and developmental potential into blastocysts of the post-warm oocytes after somatic cell nuclear transplantation was examined. Cumulus-oocyte complexes were matured, denuded, and enucleated as described previously (Parnpai *et al.*, 1999, Buffalo J 3, 371–384). The presumptive metaphase-II (M-II) oocytes before and after enucleation were first equilibrated in 7.5% DMSO + 7.5% ethylene glycol + 20% FCS in TCM199 for 10 min, and then exposed to 15% DMSO + 15% ethylene glycol + 0.5 M sucrose + 20% FCS in TCM199 for 1 min. Five oocytes were placed on a Cryotop sheet (Kitazato Supply Co., Tokyo, Japan) and vitrified in liquid nitrogen. The samples were warmed in 0.5 M sucrose solution for 5 min, directly transferred into TCM199 + 20% FCS, and kept at room temperature for 1 h before being used for a cloning experiment. The post-warm oocytes were fused with ear skin fibroblasts by two DC pulses (26 V, 17 μs) and activated with 7% ethanol for 5 min and then 10 μg/mL cycloheximide and 1.25 μg/mL cytochalasin-D for 5 h. The reconstructed embryos were cultured in mSOFaa + 0.2% BSA + 0.1% linoleic acid albumin for 2 days, and then co-cultured with bovine oviductal epithelial cells for an additional 5 days. Post-warm morphological survival of M-II oocytes (80%, 187/235) was similar to that of enucleated oocytes (75%, 158/212). Vitrified M-II oocytes were successfully enucleated (96%, 136/142) as were fresh control oocytes (88%, 143/162). Fusion rates of

M-II oocytes vitrified before and after enucleation (81%, 94/116 and 78%, 106/136, respectively) were also similar to those of fresh oocytes (81%, 100/123). Percentages of reconstructed embryos developing into hatching blastocysts on Day 7 were 5% (5/91), 6% (6/103), and 8% (8/99) in the groups of oocytes vitrified before and after enucleation, and of fresh control oocytes, respectively (ANOVA tests were not significant different). These results indicate that swamp buffalo oocytes cryopreserved by ultra-rapid vitrification procedure can be used successfully for subsequent somatic cell nuclear transplantation. (Supported by Thailand Research Fund and R&D Fund of Suranaree University of Technology)

117 EFFECTS OF PREVIOUS PRESSURE TREATMENT ON THE SURVIVAL AND DEVELOPMENTAL SPEED OF EXPANDED MOUSE BLASTOCYSTS FROZEN RAPIDLY (PILOT STUDY)

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It has been demonstrated that embryos can survive exposure to a substantial amount of pressure. (Pribenszky *et al.*, 2003 Theriogenology 59, 329, and 2002 Theriogenology 57, 506). Other studies report that, if a biological system is challenged by certain stresses, its ability to react and survive other stresses can be improved. The aim of our present study was to examine whether the survival rate of expanded mouse blastocysts could be improved by a certain pressure treatment before the freezing procedure. Morula stage mouse embryos were collected and cultured at 37°C with 5% CO_2 and maximal humidity in air in G 2.2 medium (Vitrolife, Göteborg, Sweden) to the expanded blastocyst stage. Embryos were randomly allocated to three groups. Embryos in Group I were equilibrated for 5 minutes in a solution containing 1.5 M ethylene glycol (EG) and 0.25 M sucrose in M2 (Sigma, St. Louis, MO, USA), supplemented with 10% FCS (Sigma), andthen transferred into a vitrification solution (7 M EG, 0.5 M sucrose in M2 with 10% FCS) pre-loaded in a 0.25-ml plastic straw (7–9 embryos/straw). After 1-min exposure to the vitrification solution, the straw was slowly immersed in liquid nitrogen. Embryos in Group II were loaded into 0.08-mL straws (7–9 embryos/straw) with M2. Straws were placed into the chamber, filled with M2, of a special laboratory-made device that is capable of generating and precisely detecting hydrostatic pressure up to 150 MPa (1500 atm), and were exposed to 60 MPa pressure for 30 min. After the pressure treatment, embryos were frozen as described above. Straws were thawed by transfer into 30°C water for 30 s and then the embryos were recovered and placed in rehydration medium (0.5 M sucrose in M2 supplemented with 10% FCS) for 5 min. Embryos then were cultured in medium G2.2 as described above. A total of 27, 29 and 26 embryos were assigned to Group I, Group II and the untreated control group, respectively. Embryo viability and development were assessed at 6 and 20 h after culture as determined by morphological appearance and hatching. At 6 h, 16% (4/27) of the non-pressurized embryos were one-half expanded, at 20 hours 37% (10/27) were two-thirds and 30% (8/27) were one-half expanded; none of them were hatching. While at the pressure treated groups 89% (26/29) of the embryos were fully expanded at 6 hours, and 68% (20/29) were hatching at 20 h (untreated: 25/26 fully expanded at 6 h, 24/26 hatched at 20 h). Data were analyzed by chi-square test. We considered embryos which were at least two-thirds expanded. After 6 hours Group I differed from Group II and the control ($P < 0.01$). There was no significant difference between Group II and the control ($P < 0.01$). After 20 hours the same relations were seen. In the case of hatching, Group I differed from Group II and the control ($P < 0.01$). There was no significant difference between Group II and the control ($P < 0.05$). According to our results, the applied pressure treatment improved the in vitro development of the embryos after freezing. The re-expansion was faster and the survival rate was higher for those embryos that received pressure treatment before cryopreservation. Further experiments are needed to confirm and explore the in vitro and in vivo effects and benefits of pressure treatment before freezing.

118 VITRIFICATION OF PRONUCLEAR-STAGE MOUSE EMBRYOS ON ALUMINUM FOIL FLOATING ON LIQUID NITROGEN

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The cryopreservation of pronuclear-stage embryos has a special importance in transgenic technology, cloning, and human-assisted reproductive technology. The objective of this study was to investigate the efficiency of a vitrification method modified in our lab for pronuclear-stage embryos. In experiment I, groups of 10 pronuclear-stage mouse embryos were cultured in 20 μL drops of three different culture media (G1.3/G2.3, CZB and M16) covered with mineral oil (Sigma M-8410, St. Louis, MO, USA). Twenty-four hours later, embryos cultured in G1.3 were transferred into G2.3 medium. In experiment II, 25–30 pronuclear-stage embryos were transferred into a 50-μL drop of equilibration medium containing 4% ethylene glycol (EG, Sigma E-9129) in TCM-199 (Sigma M-2520) supplemented with 10% FCS at 37°C for 12–15 min; then they were rinsed three times in 30-μL drops of vitrification medium containing 35% EG, 5% polyvinylpyrrolidone (PVP, Sigma P-0930) and 0.4 M trehalose (Sigma T-0167) in TCM-199 supplemented with 10% FCS at 37°C for 20–30 s. Embryos rinsed in vitrification solution were aspirated into a micropipette as a 1–2-μL drop containing 25–30 embryos and dropped onto aluminum foil floating on liquid nitrogen (LN_2). Vitrified droplets were stored in cryovials in LN_2. Warming was performed by moving the vitrified droplets into 0.3 M trehalose in TCM-199 supplemented with 10% FCS at 37°C. Embryos having normal morphological appearance under stereomicroscope examination were cultured in G1.3/G2.3 medium. Differences in the two experiments were analyzed by one-way ANOVA. In experiment I, development rates to the blastocyst stage were 26%, 10% and 4% for G1.3/G2.3, CZB and M16 media, respectively. The highest development rate in experiment I was obtained in G1.3/G2.3 culture media ($P < 0.05$). Therefore, G1.3/G2.3 media were used for culturing of vitrified-warmed and control embryos. In experiment II, the rate of embryos having normal morphology was 98.5%. There were no significant differences between the development rates of vitrified (13.1%) and control (18.7%) embryos to the blastocyst stage ($P > 0.05$). Although the vitrification method resulted in a high survival rate based on the morphological appearance, developmental rates of vitrified and control embryos were found to be lower than expected and reported previously by other researchers. We believe that the low developmental rates in this study were due to our culture conditions but not our vitrification method. Therefore, it could be concluded that this vitrification method is an efficient one for pronuclear-stage embryo cryopreservation and better development rates could be obtained by improving the culture conditions. This study was supported by a grant from TUBITAK, Turkey (VHAG-1908-102V048). F. Ergin is a young volunteer researcher.

119 CRYOPRESERVATION OF BIOPSIED BOVINE EMBRYOS PRODUCED IN VITRO USING ARABINOGALACTAN AND 1.5 M ETHYLENE GLYCOL

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Arabinogalactan (AG) in combination with 1.5 M ethylene glycol (EG) has been used successfully in cryopreserving biopsied in vivo bovine embryos (Darrow, 2002 Theriogenology 57(1), 531). This study was undertaken to investigate the efficiency of AG addition in a freezing medium (FM) to cryopreserve biopsied bovine embryos produced in vitro (IVP). Blastocysts of grade 1 were collected at Days 7 and 8 post-insemination. After biopsy with a small blade, embryos were transferred to CR1aa medium and cultured for 2 hours (h) before being frozen. In experiment 1, a group of unbiopsied embryos were handled in a manner similar to that used for the biopsied embryos. Embryos were frozen using either 1.5 M EG + 0.1 M sucrose (EG +) (AB Technology, Pullman, WA, USA) or a FM containing 1.5 M EG and different concentrations of AG (AG1, 2 and 3, courtesy of AB Technology). Embryos remained in FM for 10 (exp.1), 5 (exp.2), 5 and 10 (exp.3) or 5, 10, and 20 (exp.4) minutes before being loaded into a freezer and cooled down to −35°C at 0.3°C/min. Frozen embryos were thawed (35°C, 20 seconds) and cultured in CR1aa at 38.5°C for 3 days. Embryo survival rates (S%) were recorded at 24, 48 and 72 h post-thawing. Data were compared with *t*-test or ANOVA procedures using SigmaStat 3.0. Results from exp.1 (Table) indicate that biopsied and unbiopsied embryos survived well in EG + or AG2. While the biopsy procedure did not affect the post-thaw S% of embryos in either FM, no significant differences were observed between embryos frozen with EG + and AG2 ($P = 0.055$). Reducing or increasing AG concentration in FM by 2-fold (AG1 and 3, respectively) did not significantly affect the post-thaw S% at 24 h (EG +, 80.0%, $n = 133$; AG1, 83.3%, $n = 135$; AG2, 71.4%, $n = 137$ and AG3, 75.0%, $n = 135$; $P = 0.217$, exp.2). However, shortened exposure from 10 to 5 minutes to AG2 resulted in an improvement in S% at 24 h, from 35.7% ($n = 80$) to 61.4% ($n = 82$, $P < 0.05$; exp.3). When AG1 ($= 0.5 \times$ AG2) was used in the FM the S% at 24 h after different exposure times was not significant (5 minutes, 77.8%, $n = 179$; 10 and 20 minutes, 66.7%, $n = 179$ and 183; $P = 0.472$, exp.4). This study demonstrates that addition of AG to the FM effectively sustains the viability of biopsied IVP embryos during freezing and any potential harmful impact of AG on embryo survival can be minimized by reducing AG concentration or the time of embryo exposure to AG prior to freezing. Further studies are needed to determine optimal AG concentration. Currently, field trials are underway to evaluate the ability of AG medium to promote pregnancies from frozen, biopsied IVP embryos.

Table 1. Post-thaw survival rates of biopsied IVP embryos frozen in ethylene glycol with sucrose (EG +) and a FM containing arabinogalactan (AG2). Data are means ± SEM

FM	BS	*n*	24S%	48S%	72S%
EG +	U	107	83.4 ± 3.6	60.5 ± 8.7	47.9 ± 9.6
EG +	Y	105	80.1 ± 4.3	65.8 ± 6.8	42.6 ± 10.5
AG2	U	106	72.0 ± 8.3	49.1 ± 10.9	42.2 ± 10.4
AG2	Y	111	66.3 ± 7.9	44.6 ± 8.9	30.2 ± 8.2

BS: Biopsy; *n*: number of embryos cultured; 24S%, 48S% and 72S%: survival rate at 24, 48 and 72 hours post-thaw; U: unbiopsied; Y: biopsied

120 PREGNANCY RATES RESULTING FROM TRANSFER OF FRESH AND FROZEN HOLSTEIN AND JERSEY EMBRYOS

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Although it has not been documented in published studies, embryo transfer (ET) practitioners have suggested that embryos from Jersey (JE) cattle do not survive freezing as well as embryos from other dairy breeds such as Holsteins (HO). The present study represents a retrospective analysis of pregnancy rates achieved following transfer of fresh and frozen embryos from Jersey and Holstein donors. In addition, a retrospective comparison was made of two different embryo-freezing protocols for each breed of cattle. Embryos were collected nonsurgically 7 to 7.5 days post-estrus from superovulated donors on 57 Holstein and 27 Jersey dairy farms over a 15-year period. Fresh and frozen-thawed embryos were transferred nonsurgically into cows and heifers following either natural or prostaglandin-induced estrus. Embryos were frozen either in 10% glycerol (Gly) or 1.5 M ethylene glycol (EG) in 0.25 mL straws. Following equilibration, straws were seeded at −6 to −7°C and temperature was maintained for 10 min and then decreased at 0.6°C per min. Straws were plunged into liquid nitrogen at −32 to −35°C. At thawing, straws were held in the air for 7 s and then submerged in 29°C water for 15 s. Embryos frozen in EG were transferred immediately following thawing. Embryos frozen in Gly were rehydrated in a standard 3-step Gly-sucrose system prior to being transferred. Pregnancy diagnosis was performed at Days 40 to 90 of gestation. As seen in the Table 1, pregnancy rates were similar for fresh embryos from both HO and JE cattle. Also, there were no differences in pregnancy rates between recipients that received embryos frozen in Gly or EG within donors of either breed. However, JE embryos frozen in either Gly or EG resulted in lower pregnancy rates than did HO embryos frozen in Gly or EG. Embryo stage at freezing was tracked for EG but not Gly embryos. There were no differences in pregnancy rates among morulae, early blastocysts or mid-blastocysts for either HO or JE embryos frozen in EG. The differences

in embryo survival may be due to different lipid composition of embryos of the two breeds. Perhaps a more efficacious freezing protocol can be developed for cryopreservation of JE embryos. In conclusion, pregnancy rates with cryopreserved HO embryos were higher than with JE embryos.

Table 1.

Embryos	Holstein embryos		Jersey Embryos	
	No. Transfers	% Pregnant	No. Transfers	% Pregnant
Fresh	3376	65.3	1161	62.8
Frozen-Gly	836	57.7[a]	593	46.2[b]
Frozen-EG	1111	53.8[a]	193	39.9[b]

[a,b] Values in rows without common superscripts differ significantly ($P < 0.001$), chi-square analysis.

121 VITRIFICATION OF MOUSE MORULAE BY A NEW METHOD: PULLULAN FILM-STRAW VITRIFICATION

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Recent technical improvements have resulted in higher cryosurvival of oocytes and embryos of various species. However, almost all methods require thawing and washing the embryos under microscopic observation and, therefore, cannot be conveniently used for large animal ET in the field. The purpose of the present work was to develop a new embryo cryopreservation method using a water-soluble film made of pullulan (Hayashibara, Okayama, Japan) that might, in the future, be readily adaptable to field conditions. Morula-stage mouse embryos were collected from superovulated ICR donors 72 h after hCG injection. Embryos were first exposed to 10% DMSO + 10% ethylene glycol (EG) in DPBS + 20% FCS (mPBS) for 2 min, and then equilibrated for 30 s in a vitrification solution composed of 20% DMSO + 20% EG + 0.6 M sucrose in mPBS. In the pullulan film-straw vitrification method, the embryos were loaded onto the pullulan film (20 μm thick, 5 mm long and 1 mm wide) and were directly plunged into LN_2. The pullulan film was inserted into a pre-frozen 0.25 mL plastic straw (<−150°C) containing 0.15 mL mPBS and sealed with a plastic screw cap. For thawing, the medium in the straw was rapidly warmed in 37°C water while the pullulan film remained frozen by placing the top of the straw in contact with a cold iron block (<−150°C). As soon as the medium thawed, the pullulan film was immersed in the medium by a rapid downward swinging of the straw. Five min later, embryos were recovered from the straw and washed for 2 min in mPBS, for 2 min in 0.1% BSA-PBS and for 2 min in KSOM sequentially, and then cultured at 37°C in 5% CO_2 for 38 h. Noncryopreserved embryos and embryos cryopreserved by the cryoloop method (Lane *et al.*, 1999 Nat. Biotech. 17, 1234) served as controls. Data were analyzed by χ^2 test and Student's *t*-test. Results are shown in Table 1. There are no significant differences ($P > 0.05$) in either developmental abilities or cell numbers between vitrified and non-vitrified embryos. This study demonstrates that mouse morulae can be successfully vitrified and thawed by the PFSV method. This method may eventually be applied to bovine ET under field conditions.

Table 1. Development of mouse morulae in culture following vitrification by the PFSV method

Vitrification method	No. of blastocysts developed (%)	Cell numbers (mean ± SEM)
Cryoloop	28/30 (93%)	71.0 ± 1.6
PFSV	33/37 (89%)	71.3 ± 2.4
Non-vitrified control	32/35 (91%)	67.3 ± 1.6

122 COMPARISON OF TWO CRYOPROTECTANT DILUTION TREATMENTS FOR QUICK FROZEN IN VITRO-PRODUCED BOVINE EMBRYOS

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The aim of this study was to compare the viability of in vitro-produced bovine embryos following quick freezing in ethylene glycol (EG) and subsequent dilution of EG by either a two- or a three-step procedure. Cumulus-oocyte complexes (COCs) were collected from slaughterhouse ovaries. COCs were matured in TCM199 containing 10% bovine fetal serum, LH, FSH and E2, and fertilized. Presumptive zygotes were co-cultured in TCM199 with a granulosa cell monolayer, at 39°C in humidified atmosphere of 5% CO2 in air. Grade 1, expanded blastocysts ($n = 544$) were selected 7 and 9 days after insemination and randomly distributed to one of three EG equilibration treatment groups. Embryos were exposed to 10% EG for 10 min, and then to 17%, 22% or 28% EG for 30 s (respectively referred to as EG 17, EG 22 and EG 28). In all treatment groups, EG solutions were prepared in PBS + 0.2% BSA, and embryos were exposed to EG solutions at 22°C. Embryos were loaded into 0.25-mL straws which were then heat-sealed. Straws were cooled in liquid nitrogen vapor for 2 min, and then plunged and stored in liquid nitrogen. Straws were thawed in room temperature air for 10 s, and then in 25°C water for 20 s. The thawed embryos of the EG 17, EG 22 and EG 28 groups were randomly assigned to one of two EG dilution procedures. Two-step dilution consisted of transfer of embryos into PBS + 0.2% BSA + 0.3 M sucrose solution for 3 min, and then PBS + 0.2% BSA for 3 min. Three-step dilution consisted of transfer of embryos into PBS + 10% EG + 0.2% BSA + 0.3 M sucrose for 3 min, PBS + 0.2% BSA + 0.3 M sucrose for 3 min, and then PBS + 0.2% BSA for 3 min. Embryos were co-cultured on a granulosa cell

monolayer in TCM199 and evaluated after 24 h for blastocyst re-expansion (EXP), and again at 48, 72 and 96 h for hatching (HAT). A total of 724 in vitro-produced bovine blastocysts were used as controls to determine hatching rates. The results are presented in the Table. No significant differences were found between the two- and three-step dilution procedures ($P > 0.05$) for in vitro-produced bovine embryos cryopreserved by quick freezing. This project was supported by FAPESP (01/11266-4).

Table 1. In vitro re-expansion and hatching rates (%) of rapidly frozen embryos after two- or three-step dilution

Dilution	EG17		EG22		EG28		Control HAT
	EXP	HAT	EXP	HAT	EXP	HAT	
Two steps	15.4 (91) ^{Aa}	2.2 (91) ^{Aa}	24.2 (95) ^{Aa}	12.7 (95) ^{Ab}	21.7 (83) ^{Aa}	7.2 (83) ^{Aab}	70.2 (724) ^{Ac}
Three steps	15.6 (77) ^{Aa}	6.5 (77) ^{Aa}	28.7 (101) ^{Aab}	9.9 (101) ^{Aa}	30.9 (97) ^{Ab}	11.3 (97) ^{Aa}	70.2 (724) ^{Ab}

Values with different superscripts (^{A,B}) within columns differ, $P < 0.05$. Values with different superscripts (^{a,b,c}) within rows for EXP or HAT differ, $P < 0.05$, chi-square test. EXP = Re-expansion. HAT = Hatching. ( ) = number of treated embryos.

123 VITRIFICATION OF IN VITRO-PRODUCED BOVINE BLASTOCYSTS BY ADDITION OF CRYOPROTECTANT IN ONE STEP

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Vitrification combined with in-straw dilution may replace conventional cryopreservation of bovine embryos, but this requires further study for practicality. Our objectives were to compare three ethylene glycol concentrations (6, 7, and 8 M) and two equilibration times (2.5 and 3.5 min) for one-step addition of cryoprotectant. In vitro-matured oocytes from slaughterhouse ovaries, fertilized using sperm of 3 bulls, were cultured in chemically defined medium (CDM-1/CDM-2) plus FAF-BSA to produce 420 blastocysts. Day 7.5 embryos were placed into HCDM-2 (HEPES-buffered medium) and then transferred to a 6 μL drop of vitrification solution (V) (6, 7, or 8 M ethylene glycol, 0.5 M galactose, and 18% w/v Ficoll 70 in HCDM-2). Immediately thereafter, 1 cm column of DHCDM (0.5 M galactose in HCDM-2) was drawn into a 0.25 mL straw, followed by a 0.5 cm column of air and another 7 cm of DHCDM. Another 0.5 cm column of air was aspirated before the 6 μL of V (0.5 cm) containing the embryos were aspirated; then 0.5 cm of air followed. Finally, DHCDM was drawn until the first column came into contact with the cotton plug. Straws were then heat-sealed and plunged into liquid nitrogen slightly above the embryos after 2.5 or 3.5 min equilibration. The rest of the straw was then submerged slowly. Straws were thawed in air for 10 s and then in 37°C water for 20 s. Straws were held at room temperature (24°C) for 4 min before being expelled into HCDM-2. They were then placed into CDM-2 + 5% FCS for culture. Quality score (1 = excellent, 2 = fair, 3 = poor), survival (S) as determined by expansion of blastocysts, and hatching (H) were assessed at 24 and 48 h post-thaw. Data from 6 replicates (2/bull) were analyzed by ANOVA after arc sin transformation of percentage data. S and H responses were calculated as a percentage of non-frozen controls in the same replicate. Control survival and hatching rates were: 24S: 90%, 24H: 50%, 48S: 90%, 48H: 72%. Quality scores at both 24 and 48 h were higher ($P < 0.05$) for 8 M than 6 M ethylene glycol (2.68 and 3.24 for 24 h; 2.55 and 3.17 for 48 h); values for 7 M ethylene glycol were intermediate. Equilibration time had no effect on embryo quality ($P > 0.1$). Neither ethylene glycol concentration nor exposure time affected survival or hatching at 24 or 48 h ($P > 0.1$). Survival rates (as a % of control) at 48 h were: 8 M: 57%, 7 M: 55%, 6 M: 36% and hatching: 8 M: 39%, 7 M: 30%, and 6 M: 21%; 2.5 min tended to be better than 3.5 min for survival at 24 h, hatching at 24 h, survival at 48 h, but not hatching at 48 h (56% and 43%, 30% and 26%, 55% and 44%, 28% and 32% respectively). Higher concentrations of ethylene glycol proved beneficial in terms of embryo quality, with the same trend for survival and hatching rates. One-step addition of cryoprotectant for vitrification shows potential for simplifying embryo cryopreservation. However, further research is needed to produce more acceptable survival rates and to study vitrification of in vivo-produced embryos.

124 CRYOPRESERVATION OF WHITE-TAILED DEER (*ODOCOILEUS VIRGINIANUS*) SEMEN

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The methods for collecting and freezing deer semen have been, for the most part, limited to two species; red deer (*Cervus elaphus*) and fallow deer (*Dama dama*) (Asher *et al*., 2000 Anim. Reprod. Sci. 62, 195–211). The object of this study was to evaluate the progressive motility and effects of a thermal stress test on white-tailed deer (WTD) semen frozen in Biladyl extender (Mini Tube, Verona, WI, USA). Semen was collected by electroejaculation from WTD bucks ($n = 7$, ages 1.5–2.5 years) during the breeding season. This trial was the second collection for one buck (#0025) and the third collection for the other 6 bucks. The bucks were immobilized with a xylazine/ketamine mixture i.m. (2 mg kg^{-1} Vedco, Inc., St. Joseph, MO, 2.2 mg kg^{-1} ketamine HCl, Fort Dodge Animal Health, Fort Dodge, IA, USA) and electroejaculated with a Pulsator IV unit (Lane Manufacturing, Denver, Co). Semen was extended 1:1 with Biladyl A, and then slowly cooled to 4°C. Once cooled, semen was extended with equal amounts of Biladyl part A, then part B, to a final concentration of 160×10^6 cells/mL. The extended semen was then loaded into 0.25-cc straws, placed over liquid nitrogen (LN_2) in vapors (−80°C) for 10 min, and then plunged into LN_2. Straws were stored in a LN_2 tank for 3 months.

Semen was thawed in a 38.5°C water bath for 45 s, then placed in a warm test tube and incubated at 38.5°C for 5 min before progressive motility was evaluated using a computer program (Sperm Vision, Mini Tube). A thermal stress test was performed by incubating thawed samples at 38.5°C for 1 h. Results of the stress test were graded as either passed (progressive motility ≥50%) or failed (progressive motility < 50%). Results are shown in the table below. Our results show that the protocol described above is suitable for the cryopreservation of white-tailed deer semen. These data suggest that the initial post-thaw progressive motility may not accurately represent the potential progressive motility of the spermatozoa (e.g. WTD s 0038 & 0103).

Table 1. Volume collected and post thaw evaluation of white-tailed deer semen

ID#	Vol. semen collected	Post thaw progressive motility (%)	Stress test
0010	0.80 mL	74	Passed
0024	1.10 mL	70	Passed
0025	2.75 mL	50	Failed
0038	100 μL	34	Passed
0041	2.00 mL	48	Failed
0103	1.00 mL	42	Passed
0112	400 μL	74	Passed