

76 PRODUCTION OF CLONED BOVINE TRANSGENIC EMBRYOS WITH VARIOUS TYPES OF MONO-COLONY CELLS AND OVUM PICKUP

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The objective of this study was to determine the effects of genetic manipulation, cell type, and culture conditions on developmental potential of bovine nuclear transfer (NT) embryos. Ovum pickup (OPU) technology was developed to obtain the oocytes for NT. A total 4044 cumulus-oocyte complexes (COCs) were obtained during 492 OPU sessions, with an average of 8.2 COCs recovered each session. Cultured granulosa cells (CGC), bovine fetal (150 days) oviduct epidermic cells (FOEC), and adult ear skin fibroblasts (ASFC) were used as donor cells for NT and were transfected with the expression vector including human FIX coding sequence directed by goat β -casein promoter and neomycin gene. The cells were screened under $800 \mu\text{g mL}^{-1}$ G418 for 10–14 days until the appearance of a “mono-colony” of cells which were then picked. Each cell population was expanded by consecutive passage culture under $300 \mu\text{g mL}^{-1}$ G418 until used for NT, ensuring that the majority of cells were transgenic. Oocytes were enucleated at 20 h post-maturation and a single donor cell was transferred into the perivitelline space of a recipient oocyte. After fusion and activation, the reconstructed embryos were co-cultured with vero cells in B2 medium for 7 days. NT efficiency between primary granulosa cells (PGC) without in vitro culture and CGC, as well as among CGC, FOEC and ASFC that were transfected with exogenous DNA (named TCGC, TFOEC, TASFC, respectively), were compared (Table 1). Differences between groups were verified by chi-square test using SAS 6.12 (SAS Institute, Inc., Cary, NC, USA) program. CGCs presented a higher fusion rate ($P < 0.01$) for reconstructed embryos and higher development to the blastocyst stage for NT embryos than did PGC (67% vs. 54% and 41% vs. 21%, respectively). There were no significant differences ($P > 0.05$) in cleavage rate (65%, 71%, and 69%, respectively) and development to the blastocyst stage for NT embryos (36%, 30% and 40%, respectively) for TCGC, TFOEC, and TASFC. A total of 86 blastocysts were selected for transfer into uteri of 86 cows, resulting in 26 pregnancies (30%) at 60 days by ultrasound scanning. Among these, 12 cows remain pregnant and 14 have aborted. The results indicated that oocytes recovered from OPU can be successfully used for NT with development to the blastocyst stage. PGC, CGC, FOEC, and ASFC can all be used for generating transgenic cattle by NT, although this needs to be verified by the birth of live calves.

Table 1. Nuclear transfer efficiency with various cell types

Donor cell	Oocytes, <i>n</i>	Maturation (%)	Karyoplast–cytoplasm complexes, <i>n</i>	Fused (%)	Culture	Cleaved Day 2 (%)	Blastocysts Day 7 (%)
CGC	950	773 (81) ^a	638	425 (67) ^{a,b}	335	232 (69) ^a	95 (41) ^a
PGC	787	635 (81) ^a	525	281 (54) ^a	178	106 (60) ^a	37 (21) ^a
TCGC	735	580 (79) ^a	486	301 (62) ^{c,d}	234	152 (65) ^a	55 (36) ^a
TFOEC	398	336 (84) ^a	298	121 (41) ^c	114	81 (71) ^a	24 (30) ^a
TASFC	289	230 (80) ^a	204	115 (56) ^{b,d}	91	63 (69) ^a	25 (40) ^a

^{a,b,c,d} Values with the same superscript within a column are not significantly different.

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Cryopreservation/Cryobiology

77 FREEZING OF MOUSE SPERM BY THREE DIFFERENT CRYOPROTECTANTS

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The cryopreservation of sperm has contributed greatly to animal breeding and reproduction. This study was designed to examine the effect of raffinose, sucrose, and trehalose as cryoprotectants for freezing of mouse sperm. The cryoprotectant solution (CPA) consisting of 3% skim milk (Skim Milk dehydrated, Bacto, Difco, Seoul, Korea) as buffer or extender was prepared and supplemented with 0.3 M raffinose (D[+]-raffinose pentahydrate, Sigma) or sucrose or trehalose as non-permeating cryoprotectants. Sperm samples for cryopreservation were collected from caudae epididymides and vas deferens of males of four mouse strains (ICR, FVB, C57BL/6, and CBA). Sperm samples from individual males were aliquoted in cryotubes, placed immediately in the vapor phase of a liquid nitrogen storage container for 10 min, and then plunged directly in liquid nitrogen. For thawing, frozen cryotubes were removed from liquid nitrogen and placed directly into a water bath kept at 37°C for approximately 2 min until the ice melted. Survival of mouse sperm was measured by vital staining. Survival rates of spermatozoa frozen and thawed in freezing solution supplemented with raffinose were higher (ICR: 51.0%, FVB: 27.6%, C57BL/6: 25.7%, and CBA: 23.3%) than those supplemented with sucrose (35.8, 19.6, 12.3, and 19.7%) or trehalose (16.0, 25.4, 25.3, and 24.7%), all respectively. Furthermore, *in vitro* fertilization of mouse oocytes with sperm frozen in raffinose gave cleavage rates of 61.1 (FVB: 217/355 eggs), 59.4 (C57BL/6: 165/278 eggs), and 57.1% (CBA: 144/252 eggs), respectively. These cleaved embryos (FVB: 163, C57BL/6: 137, and CBA: 134) were transferred to 7, 5, and 6 pseudopregnant females, respectively. A total of 4, 3, and 4 females in the respective strains became pregnant and delivered 17 (10.4%), 17 (12.4%), and 18 (13.4%) offspring, respectively. Our results suggest that raffinose is a good cryoprotectant for freezing of sperm for production of inbred mice.

78 SURVIVAL OF PORCINE OOCYTES AT GERMINAL VESICLE STAGE AFTER VITRIFICATION WITH OPEN PULLED STRAW METHOD

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The present study was performed to assess the survival of immature denuded or cumulus-covered porcine oocytes (COCs). Immature porcine oocytes were collected from 2–6 mm follicles of slaughterhouse ovaries and subjected to open pulled straw (OPS) vitrification, according to the method of Vajta *et al.* (1998 Mol Reprod. Dev. 51, 53–58). After vitrification, oocytes were matured *in vitro* for 48 h at 39°C, 5% CO₂ in air. The maturation medium was TCM199 supplemented with 10% pig follicular fluid, 1.25 mM L-glutamine, 0.9 mM Na pyruvate, 150 µM cysteamine, 0.1 mg/mL streptomycin sulfate, 100 IU/mL PG penicillin G potassium, 10 IU/mL PMSG, and 25 IU/mL hCG. After IVM, to assess nuclear stage, all oocytes were fixed with acetic acid–alcohol (1 : 3) for at least three days and then stained with 0.1% orcein and examined under a phase-contrast microscope at 100× magnification. All data were analyzed by χ^2 test ($P < 0.05$). Immediately after collection, all oocytes were at the germinal vesicle (GV) stage with an intact GV membrane. After vitrification, significantly fewer oocytes had normal morphology (intact plasma membrane) in the denuded and COC groups (4.7% and 8.5%, respectively) than did the denuded and COC control groups (95% and 92%, respectively). By the end of IVM, significantly fewer oocytes were surrounded by expanded cumulus after vitrification of COCs than were the COC controls (28.1% and 63.5%, respectively). After IVM, more of the COC control oocytes underwent germinal vesicle breakdown than did the denuded controls (95% and 78.2%, respectively); the rate of MII oocytes was higher for the COC controls than for the denuded controls (80% and 54.5%, respectively). After vitrification, the number of oocytes that underwent GVBD was significantly less for both the denuded and the COC groups (2.0% and 7.0%, respectively); the percentage of oocytes that reached MII was also lower (0.64% and 2.78%, respectively). Most of the vitrified oocytes had a damaged GV with disrupted membrane and cluster-like or scattered chromatin in both the denuded and the COC groups (96.4% and 90.7%, respectively). These data suggest that vitrification of cumulus-enclosed immature porcine oocytes is preferable compared to vitrification of denuded ones. Loss of cumulus cells compromises competence of oocytes to resume meiosis, which might result in a lower maturation rate after IVM.

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79 EFFECT OF PRE-FREEZE ADDITION OF PLATELET-ACTIVATING FACTOR AND PLATELET-ACTIVATING FACTOR:ACETYLHYDROLASE ON THE POST-THAW INTEGRITY OF FROZEN-THAWED BOAR SPERM

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The use of frozen-thawed boar sperm is not widespread, owing to reduced fertility rates and high cost per dose (Eriksson *et al.* 2004 Proc. Aust. Assoc. Pig Vet., 61–69). Improvements in post-thaw sperm survival are required for commercialization. Platelet-activating factor (PAF) is a phospholipid involved in regulating sperm function. PAF:acetylhydrolase (PAF:AH) regulates PAF activity by conversion to its inactive isoform. Both occur naturally in boar semen (Kordan *et al.* 2003 Pol. J. Vet. Sci. 6, 55–60). Removal of PAF and PAF:AH along with seminal plasma during the cryopreservation process may inhibit the ability of sperm to withstand the freeze-thawing process. The aim of this study was to assess the effect of PAF and PAF:AH, added to boar semen pre-freeze, on the post-thaw motility and acrosome integrity of sperm. The sperm rich fraction was collected from a mature Large White × Landrace boar, diluted with Androhep (1 : 2, semen:Androhep; Minitube, Verona, WI, USA), cooled to 17°C over 2 h, and then centrifuged (10 min, 800g). The sperm pellet was resuspended in cooling extender (11% (w/v) lactose solution with 20% (v/v) egg yolk; control), cooling extender plus 100 ng/mL PAF (PAF), or cooling extender plus 0.4% (v/v) PAF:AH (Pafase; ICOS Corporation, Seattle, Washington, USA), and cooled to 5°C over 2.5 h. Sperm were further diluted with cooling extender plus 9% (v/v) glycerol and 1.5% (v/v) Equex STM (freezing extender), loaded into 0.5-mL straws, and frozen. Straws were thawed (20 s, 42°C) and the motility and acrosome integrity (FITC-PNA; Mortimer *et al.* 1990 Hum. Reprod. 5, 99–103) assessed at 0, 3, and 6 h post-thaw after incubation at 37°C. Data from three replicates were analyzed by ANOVA and a Tukey test applied where significant differences were found. Post-thaw motility (0 and 3 h) was higher for PAF (60.0 ± 0.0% and 25.0 ± 2.9%) than for control (41.7 ± 1.7% and 10.0 ± 2.9%; $P < 0.05$), but was similar for Pafase (41.7 ± 1.7% and 16.7 ± 1.7%; $P > 0.05$). By 6 h post-thaw, motility was similar for PAF (1.7 ± 1.7%), Pafase (6.7 ± 6.8%), and control (1.7 ± 1.7%, all respectively; $P > 0.05$). Acrosome integrity was higher at 0, 3 and 6 h post-thaw for Pafase (55.7 ± 3.2%, 45.7 ± 3.7% and 23.0 ± 3.1%) than for control (42.7 ± 1.5%, 25.7 ± 5.7% and 12.3 ± 2.7%) and PAF (33.0 ± 3.7%, 26.3 ± 2.2% and 11.7 ± 0.3%, all respectively; $P < 0.05$), but was similar between control and PAF ($P > 0.05$). Supplementation of cooling extender with 100 ng/mL PAF increased initial post-thaw motility, but this benefit was lost after 6 h post-thaw. Pafase in the cooling extender improved the proportion of intact acrosomes, even after 6 h post-thaw. *In vitro* studies investigating the interaction between Pafase-treated frozen-thawed sperm and oviducal epithelial cells would be of interest to further establish the potential benefits of pre-freeze addition of Pafase on the fertilizing potential of frozen-thawed boar sperm.

80 GENE EXPRESSION PROFILES AND *IN VITRO* DEVELOPMENT FOLLOWING VITRIFICATION OF PRONUCLEAR AND 8 CELL-STAGE MOUSE EMBRYOS

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The analysis of differences in gene expression of embryos in response to cryopreservation may explain some of the observed differences in further development. Experiments were conducted to study effects of two vitrification methods, solid surface (SSV) and in-straw vitrification (STR), for pronuclear and 8 cell-stage mouse embryos with regard to gene expression and *in vitro* development. Both stages of embryos were vitrified by SSV (Dinnyes *et al.* 2000 Biol. Reprod. 63, 513–518) using 35% ethylene glycol (EG) for vitrification solution (VS) and STR using 40% EG for VS. Warming and rehydration of embryos in the SSV method was performed by placing vitrified droplets into a 37°C 0.3 M trehalose solution for 1 min, and then for 2 min in each of 0.15 M trehalose, 0.075 M trehalose, and base medium at room temperature before culture in CZB medium. Warming of embryos for the in-straw method was performed by holding straws in air for 10 s and then plunging them into 37°C water for 15–20 s. The contents of the straws were expelled into 37°C 0.3 M trehalose solution and embryos were treated as above. No significant differences were found between immediate survival rates of embryos vitrified by SSV and STR in both stages. Blastocyst rates were significantly higher with SSV than with STR and not significantly different from those of the control (Table 1). These results show that SSV was more efficient than STR. Quantification of selected gene transcripts from single embryo (6 embryos/treatment group) was carried out by quantitative real-time RT-PCR. Genes related to oxidative stress (MnSOD and CuSOD), cold stress (CIRP, RBM3, and p53), and β -actin as reference gene were amplified. We found up-regulation of all stress genes at 3 h post-warming in all treatment groups. At 10 h post-warming, a low level of gene expression was found in SSV-treated embryos, but gene expression remained at high level in STR-treated embryos. However, no differences in gene expression were found between blastocysts developed from fresh and vitrified embryos. In conclusion, the real-time RT-PCR method from single embryos opened new opportunities for understanding molecular events following cryopreservation. The continuous up-regulation of stress-related genes at 10 h post-warming might have been an early indicator of reduced viability following STR, as was also indicated by the developmental rate to the blastocyst stage.

Table 1. Survival and blastocyst rates of vitrified/warmed pronuclear and 8 cell-stage mouse embryos

Embryo stages	Treatment	Survival rates (%)	Blastocyst rates (%)
Pronuclear	Control	85/85 (100) ^a	65/85 (77) ^a
	SSV	51/65 (79) ^b	31/51 (61) ^a
	STR	58/74 (78) ^b	20/58 (35) ^b
8 cell	Control	65/65 (100) ^a	58/65 (89) ^a
	SSV	58/77 (75) ^b	51/58 (88) ^a
	STR	50/76 (66) ^b	24/50 (48) ^b

^{a,b} Values differ significantly within same column in a given stage; chi-square method ($P < 0.05$).

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81 EFFECT OF SUCROSE CONCENTRATION IN WARMING MEDIUM ON THE DEVELOPMENT POTENTIAL OF VITRIFIED BOVINE OOCYTES

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The aim of this experiment was to determine the effect of the sucrose concentration (0 to 0.33 M) in the dilution medium on the viability, fertilizability, and development of vitrified bovine oocytes. Bovine oocyte-cumulus complexes were collected from slaughterhouse ovaries and *in vitro*-matured as reported previously. After 24-h maturation in TCM199-based medium under 5% CO₂ humidified air at 39°C, these were exposed to hyaluronidase and carefully pipetted to remove all except the 3–5 innermost layers of cumulus. Oocytes were put into the pre-equilibration medium for 3 min and then into vitrification solution containing HEPES-buffered TCM199 supplemented with 20% FBS, ethylene glycol, and dimethylsulphoxide for 25–30 s; they were then vitrified by modified solid surface vitrification (Dinnyes *et al.* 2000 Biol. Reprod. 63, 513–518). The oocytes were warmed at 39°C by placing them in holding medium with 0, 0.08, 0.17, 0.25, or 0.33 M sucrose. Non-vitrified oocytes were used as controls. Oocytes were inseminated 30 min after warming, and the presumptive zygotes were cultured in CR1-aa medium supplemented with 6 mg/mL BSA at 39°C in a humidified atmosphere of 5% CO₂, 5% O₂, and 90% N₂ for eight days. Data were analyzed by one-way ANOVA. As shown in Table 1, there was

no significant difference in survival rate ($P > 0.05$) of the vitrified oocytes that were placed in dilution solution containing 0.17, 0.25, or 0.33 M sucrose and the non-treated controls. On Day 2 (fertilized on Day 0), cleavage to the 8-cell stage was similar for the 0.17, 0.25, and 0.33 M dilution groups, but the rates for all three were significantly lower ($P < 0.05$) than for the control group. The blastocyst rate on Day 8 was significantly higher for the 0.25 M group than for any other experimental group but still significantly lower than for the control. In conclusion, this study suggests that with this vitrification/warming procedure the optimum concentration of sucrose in the dilution solution is 0.25 M.

Table 1. Oocyte survival after vitrification/warming and subsequent embryo development

Groups	Total	Survived (%)	8-cells (%)	Cleavage (%)	BL (%)
0	230	111 (48) ^b	31 (28) ^c	37 (33) ^b	3 (3) ^d
0.085	211	140 (66) ^{b,c}	53 (38) ^{b,c}	65 (46) ^{b,c}	7 (5) ^d
0.17	238	192 (81) ^{a,c}	88 (46) ^b	119 (62) ^{a,b,c}	28 (15) ^{b,c}
0.25	475	407 (86) ^a	182 (45) ^b	255 (63) ^{a,b,c}	88 (22) ^c
0.33	281	250 (89) ^a	103 (41) ^b	152 (61) ^{a,c}	34 (14) ^b
Control	1331	1331 (100) ^a	817 (61) ^a	1100 (83) ^a	584 (44) ^a

a,b,c,d Values within columns with different superscripts are significantly different ($P < 0.05$).

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82 EVALUATION OF CELL DEATH IN CRYOPRESERVED MOUSE EMBRYOS

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Cryopreservation of mammalian embryos is an important tool for the application of reproductive biotechnology. Recent evidence indicates that apoptosis may be important in determining the viability of cryopreserved embryos. Our goal was to detect apoptosis and characterize and quantify the embryonic cell death caused by cryopreservation. Mouse morulae were collected, selected, and separated into three groups: fresh, slow-freezing, and vitrification. In the slow-freezing procedure, embryos were exposed to 10% ethylene glycol (EG) for 10 min. After loading, the straws were placed into methanol at -7°C for 5 min, seeded and after 5 min cooled at $0.5^{\circ}\text{C}/\text{minute}$. After 10 minutes at -31°C , straws were plunged into and stored in liquid nitrogen. Slow-frozen straws were thawed in air for 10 s, and then immersed in a 25°C water bath for 20 s. Embryos were vitrified by exposing them to 10% and 20% EG for 5 min followed by 40% EG + 18% Ficoll + 10% sucrose (EFS) for 30 s and the 0.25-mL straws then plunged into and stored in liquid nitrogen. The vitrified straws were warmed by immersing them in 25°C water for 20 s. Cell membrane integrity was assessed by Hoechst and propidium iodide double staining (H/PI). Fresh and thawed embryos were scored (following IETS recommendations) and then fixed after 30 min in PBS + 10% FCS. Morphology and apoptosis were assessed with Haematoxylin-Eosin staining (HE) and by electron microscopy (MET). The number of Grade I embryos recovered after thawing was higher for slow-frozen embryos (61.5%) than vitrified embryos (29.5%). H/PI detected more membrane permeability in the vitrified embryos (69.7%), than in the slow-frozen (48.4%) or non-frozen (13.8%) groups ($P < 0.05$, Wilcoxon's test). Nuclear evaluation by HE revealed that vitrification and slow-freezing induced pyknosis and chromatin condensation. Mitotic pattern was observed in the fresh and slow-frozen group, but not in vitrification group suggesting that the embryos were either not randomly allocated to the groups or not-treated and fixed at the same age, or that vitrification changed the nuclear status of the embryos. HE staining revealed weakly staining cytoplasm and degenerated cells in the vitrification group (indicating oncosis), while in the slow-frozen group the presence of cytoplasmic condensation and eosinophilic structures indicated apoptosis. The ultrastructure examination confirmed the HE observations. In conclusion, the results demonstrated that staining with HE allows detection of oncosis and apoptosis in cryopreserved embryos. According to these data, vitrification caused more cellular injuries than slow-freezing, and oncosis was the predominant injury. It is important to point that specific molecular apoptosis tests must be performed to confirm these results.

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83 CRYOPRESERVATION OF *IN VITRO* PORCINE OOCYTES BY SOLID SURFACE VITRIFICATION (SSV)

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The effects of the cytoskeletal inhibitor cytochalasin in solid surface vitrification (SSV; Dinnyes *et al.* 2000 Biol. Reprod. 63, 513–518) were investigated on *in vitro* matured (IVM) porcine oocytes. Cumulus-free IVM oocytes were subjected to one of the following: SSV (equilibration in

4% ethylene glycol (EG) for 10 min followed by vitrification in 35% EG, 5% polyvinyl pyrrolidone, and 0.3 M trehalose on a cold (about -150°C) surface; warming in 0.4 M trehalose at 37°C), SSV pre-treatment with $5\text{ }\mu\text{g/mL}$ cytochalasin B (SSV + CB), or the steps of SSV without cooling, i.e. toxicity control (TC). Non-lysed oocytes together with the non-treated controls were subjected to parthenogenetic activation and then *in vitro* cultured (IVC) for six days. The proportion of non-lysed oocytes was higher when pre-treatment with CB was performed compared to SSV. However, both results were significantly lower than that of the TC. After parthenogenetic activation via a combination of a direct current electric pulse (1 kV/cm for $45\text{ }\mu\text{s}$) followed by 3 h treatment with 2 mM 6-dimethylaminopurine, the proportion of cleaved embryos was higher in the SSV + CB than in the SSV. Nevertheless, significantly more oocytes cleaved in the TC and control groups. On Day 6 no blastocyst, were determined in the SSV group, and one blastocyst was obtained in the SSV + CB, while significantly more blastocysts developed in both the TC and the control (Table 1). There was no significant difference in blastocyst rates and in the number of blastomere nuclei/embryo between the TC and the control. These results indicate that the high concentration of cryoprotectants per se applied in SSV were not detrimental for *in vitro* development and that CB pre-treatment increased survival and further development of SSV vitrified pig oocytes resulting in one parthenogenetic blastocyst from vitrified pig oocytes.

Table 1. parthenogenetic development of vitrified IVM porcine oocytes

Treatment	No. oocytes	No. (% $\pm$ SEM) non-lysed oocytes	No. oocytes subjected to activation	No. (% $\pm$ SEM) cleaved oocytes	No. (% $\pm$ SEM) blastocysts on Day 6 IVC
SSV	754	95 (12.7 ± 1.1) ^d	95	7 (7.5 ± 3.7) ^c	0
SSV + CB	748	171 (23.1 ± 4.4) ^c	171	24 (14.0 ± 2.3) ^b	1 (0.53 ± 0.53) ^b
TC	188	167 (87.1 ± 5.7) ^b	167	100 (55.5 ± 10.6) ^a	33 (19.74 ± 2.79) ^a
Control	199	199 (100) ^a	199	117 (59.4 ± 7.2) ^a	50 (25.52 ± 5.38) ^a

a,b,c,d Values differ significantly ($P < 0.05$; ANOVA) within each column.

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84 SUCCESSFUL CRYOPRESERVATION OF PORCINE EMBRYOS BY THE METAL MESH VITRIFICATION (MMV) METHOD

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The present study was designed to generate piglets from porcine embryos by the metal mesh vitrification (MMV) method. Prepuberal gilts were administered eCG and hCG and were artificially inseminated. Morulae and expanding blastocysts (diameter = approximately $200\text{ }\mu\text{m}$) were collected at 144 h and 168 h after hCG injection, respectively. The metal mesh and the plastic plate (control) were used as sample containers for ultrarapid vitrification. The metal mesh ($75\text{ }\mu\text{m}$ stainless steel mesh) was 1.5 mm wide and 10 mm long, and the 3 mm at the end of the mesh was bent at a right angle. Plastic plates, made from 0.25 mL plastic straws, were the same size and form as the metal mesh. Embryos were equilibrated with 7.5% ethylene glycol (EG) + 7.5% DMSO + 10% fetal bovine serum (FBS) in PBS for 5 min, followed by exposure to 15% EG + 15% DMSO + 0.6 M trehalose + 10% FBS in PBS for 1 min. Embryos were picked up on the metal mesh or loaded onto plastic plates with minimum volume of the solution, and then plunged into liquid nitrogen. Sample containers were placed in 1.8-mL cryotubes and stored in liquid nitrogen. Warming and dilution were performed by moving the container from liquid nitrogen into 0.5 M trehalose + 10% FBS in PBS at 37°C for 5 min. Embryos were rinsed twice in 4 mg/mL BSA + 10% FBS in NCSU37 (mNCSU37) for 5 min. Survival of the vitrified embryos was assessed after culture in mNCSU37 for 24 h (expanding blastocysts) or 48 h (morulae), and those that survived to fully expanded, hatching or hatched blastocysts were scored as viable. The vitrified warmed embryos were transferred surgically to recipient gilts. Experiment 1: The survival rates of expanding blastocysts vitrified by MMV or the control method were compared. The rate by MMV was significantly higher (84% ; $P < 0.01$ by χ^2 test) than that of the control (53%). Experiment 2: The developmental stage (morula or expanding blastocyst) suitable for vitrification was examined. Survival of expanding blastocysts was significantly higher (84% ; $P < 0.05$) than that of morulae (55%). Experiment 3: Expanding blastocysts were vitrified by MMV, warmed, and transferred into recipients (20 embryos per recipient). Eight of 10 recipients were found to be pregnant. Seven recipients farrowed a total of 37 live and 1 stillborn piglets. The other recipient miscarried and farrowed 4 stillborn piglets. These results showed that the viability of vitrified warmed porcine embryos was affected by the material of the sample container (Experiment 1) and also by the developmental stage (Experiment 2). Since the transferred embryos showed an excellent ability to develop into piglets (Experiment 3), the MMV method developed in the present study seems to be an effective preservation method for porcine embryos.

85 ADDITION OF REDUCED GLUTATHIONE TO FREEZING MEDIUM IMPROVED THE SPERM FUNCTIONALITY OF THAWED BOAR SPERMATOZOA

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The processes of cooling and freezing/thawing produce physical and chemical stresses on the sperm membrane that are associated with the oxidative stress and reactive oxygen species (ROS) generation that reduce sperm viability and fertilizing ability. It is known that the process of freezing

is associated with a significant reduction of the GSH content in boar sperm (Gadea *et al.* 2004 Theriogenology 62, 690–701). The addition of antioxidants to freezing medium could prevent the formation of ROS and improve the seminal parameters. The aim of these experiments was to investigate the effects of the addition of reduced glutathione (GSH) to freezing extenders on capacitation status and changes in the sulfhydryl groups of proteins on the sperm surface. Ejaculate-rich fractions from three mature boars were frozen by classic methodology (Westendorf *et al.* 1975 Dtsch. Tierarztl. Wochenschr. 82, 393–395) using lactose/egg-yolk extender with 0 mM (group 0), 1 mM (group 1), or 5 mM (group 5) GSH. To detect increase in plasma membrane lipid packing disorder, sperm samples were stained with merocyanine 540 (M540) and Yo-Pro 1 (Harrison *et al.* 1996 Mol. Reprod. Dev. 45, 378–391). Cells were classified as low M540 (viable, noncapacitated), high M540 (viable, capacitated), or Yo-Pro-1 positive (dead sperm) using flow cytometry. The sulfhydryl status of proteins from spermatozoa surface was evaluated with a fluorescent stain 5-iodoacetamidofluoresceine (5-IAF). The addition of GSH to the freezing medium had a positive influence on the parameters studied, increasing the proportion of viable noncapacitated spermatozoa and reducing the number of dead with a similar number of viable capacitated (Table 1). The proportion of spermatozoa stained by 5-IAF was significantly lower when GSH was added. In conclusion, we can assume that the addition of reduced glutathione to the freezing medium had a protective effect on spermatozoa functionality.

Table 1. Capacitation status and changes in sulfhydryl groups of proteins (5-IAF staining) after thawing of frozen boar spermatozoa

Freezing media	Viable capacitated	Viable non-capacitated	Dead	5-IAF staining
Control	8.7 ± 0.9	35.3 ± 2.6 ^a	56.1 ± 2.9 ^a	54.2 ± 1.7 ^a
1 mM GSH	9.1 ± 0.5	48.6 ± 2.3 ^b	42.3 ± 2.0 ^b	41.4 ± 1.6 ^b
5 mM GSH	8.9 ± 0.7	40.3 ± 2.2 ^{ab}	50.7 ± 1.8 ^{ab}	40.2 ± 1.5 ^b
ANOVA <i>P</i> -values	0.918	0.003	0.001	0.001

^{a,b} Values within columns with different superscripts differ (*P* < 0.05).

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86 CULTURE OF PIG EMBRYOS BEFORE CRYOPRESERVATION

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Our previous experiment on cryoconservation of *in vitro*-cultured porcine embryos (Gajda and Smorag 2000 CryoLetters 21, 231–236) revealed that, unlike for other species, sensitivity to vitrification of such embryos is higher than for those obtained *in vivo*. Considering that, selection of an optimal *in vitro* culture medium for pig embryos before cryoconservation becomes more important. Two experiments have been done on 3/4-cell pig embryos. Embryos were obtained from superovulated gilts after flushing the oviduct with PBS medium supplemented with 20% fetal calf serum at 38°C. In experiment 1, the embryos were cultured in three chemically defined media: NCSU-23, NCSU-37, CZB. The culture was performed at 39°C, 5% CO₂ in air for 96 to 120 h. The main evaluation criterion was development into blastocysts. Additionally, embryos that developed into blastocysts were stained with Hoechst 33342 and the cells were counted under a fluorescence microscope. Data were analyzed using chi-square and Fisher tests. The highest percentage of embryos developing into blastocysts was observed for those cultured in NCSU-23 (a): 89.2%; lower in NCSU-37 (b): 78.9%; and lowest for those in CZB medium (c): 66.6% (a,c: *P* < 0.05; a,b and b,c: NS). There were significantly (*P* < 0.001) more total cells in blastocysts obtained after culture in NCSU-23 (139.5 ± 32.8) than after culture in NCSU-37 (71.9 ± 36.6) or in CZB medium (58.3 ± 8.6). In experiment 2, embryos were cultured *in vitro* in NCSU-23 medium to the blastocyst stage and then vitrified (Gajda and Smorag 2002 CryoLetters 23, 385–388). Embryos were vitrified in 0.25-mL plastic straws in a mixture of 40% v/v ethylene glycol, 18% w/v Ficoll, and 0.3 M sucrose. Straws with embryos were stored in liquid nitrogen for 3 to 6 months. Dilution after rapid thawing (water bath at 20°C) was done in one step in 0.5 M sucrose solution. Eighty-five thawed blastocysts were surgically transferred into the oviducts of four synchronized recipient gilts. Two recipients became pregnant and farrowed 11 live healthy piglets. These results indicated that NCSU-23 medium provided the best conditions (of those media tested) for *in vitro* culture of pig embryos before vitrification. Transfer into oviducts of blastocysts that developed from *in vitro*-cultured and vitrified embryos resulted in full development *in vivo*.

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87 VITRIFICATION OF BOVINE OOCYTES TREATED WITH CHOLESTEROL-LOADED METHYL-β-CYCLODEXTRIN

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Addition of cholesterol-loaded cyclodextrin (CLC) can increase sperm cryosurvival (Purdy *et al.* 2000 Cryobiology 48, 36–45). The purpose of this study was to determine if cryosurvival of vitrified oocytes could be improved by incubation with CLC prior to vitrification. Slaughterhouse-derived cumulus oocyte complexes were matured in a chemically defined medium with fatty acid-free BSA and hormones for 21 h followed by

partial cumulus removal with 100 U/mL hyaluronidase and gentle pipetting. For an additional hour, oocytes were placed into maturation medium supplemented with 0.5% PVA instead of BSA with or without 2.5 mg/mL CLC. At 22 h after the start of maturation, oocytes were transferred to handling media containing 20% FCS or 0.5% PVA in TCM-199 + HEPES (HTCM-199). Oocytes with approximately 3 layers of cumulus were vitrified in two steps. First, they were exposed to VS1 (10% ethylene glycol (EG), 10% DMSO, 6% PVP, or 20% FCS, in HTCM-199) for 30 s, then exposed to VS2 (20% EG, 20% DMSO, 6% PVP, or 20% FCS, 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 through 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, fertilized with semen from 3 bulls, 3 replicates each, and cultured according to standard procedures (Zhang *et al.* 2003 *Theriogenology* 60, 1657–1663). For each replicate, 30 oocytes were assigned to the following treatments: A: chemically-defined media with PVA for the last hour of maturation, handling and vitrification; B: same as A except CLC treatment, for 1 h before vitrification; C: chemically defined media for maturation, but with 20% FCS for HM, VS1 and VS2. Data were analyzed by ANOVA. CLC treatment resulted in higher cleavage rates and 8- to 16-cell embryo production, but not higher blastocyst (bl) production (Table 1). Non-vitrified oocytes developed better than vitrified ones (means: cleavage, 76%; 8- to 16-cell, 64%; bl D8, 21%; bl D9, 24%). Further studies with vitrification of cholesterol-loaded cyclodextrin-treated oocytes and chemically defined media are warranted.

Table 1. Development of vitrified oocytes (LS means $\pm$ SE)

Treatment	% cleavage**	% 8-16-cell*	% bl Day 8	% bl Day 9
A (PVA)	55 $\pm$ 3.6 ^a	41 $\pm$ 4.2 ^a	5.5 $\pm$ 1.2	9.4 $\pm$ 1.8
B (PVA-CLC)	68 $\pm$ 3.6 ^b	55 $\pm$ 4.2 ^b	7.1 $\pm$ 1.2	9.1 $\pm$ 1.8
C (FCS)	51 $\pm$ 3.6 ^a	38 $\pm$ 4.2 ^a	4 $\pm$ 1.2	5.3 $\pm$ 1.8

^{a,b} Means without common superscripts differ ($P < 0.01^{**}$ or 0.05^{*}).

88 PREGNANCY RATES FOR *IN VITRO* AND *IN VIVO* PRODUCED OVINE EMBRYOS VITRIFIED USING THE MINIMUM VOLUME COOLING CRYOTOP METHOD

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Previously we reported that, using the minimum volume cooling (MVC) cryotop vitrification method, *in vitro*-produced ovine and bovine embryo survival after thawing was similar to that of fresh embryos (Kelly *et al.* 2004 *Reprod. Fert. Dev.* 16, 172). While survival of vitrified embryos after thawing can be indicative of embryo viability, this assessment does not always correlate with embryo survival after transfer. This study assesses the effect of vitrification using the MVC cryotop method on the survival after transfer of *in vitro*- and *in vivo*-produced ovine embryos. Fresh or vitrified Day 6 ovine embryos (expanded blastocysts, blastocysts, compact morulae) were used in this study. Ovine cumulus–oocyte complexes were obtained and matured, fertilized (Day 0), and cultured *in vitro* (Walker *et al.* 1996 *Biol. Reprod.* 55, 703–708). *In vitro* embryos for vitrification were produced and vitrified (Kelly *et al.* 2004 *Reprod. Fert. Dev.* 16, 172) 10 days prior to the day of transfer. *In vivo* embryos were recovered from donor Merino ewes and vitrified 7 days prior to the day of transfer while fresh *in vivo* embryos were collected and transferred on the same day. Semen used for both *in vivo* and *in vitro* embryo production was from the same sire. On the day of transfer, vitrified embryos were thawed directly into 1.25 M sucrose solution, followed by stepwise dilution of the cryoprotectants. Embryos were transferred as singles into synchronized recipient ewes on a randomized basis. Fetal number was detected at Day 50. Variables were assessed using the CATMOD procedure in SAS. Pregnancy rate for *in vivo*-derived embryos was higher ($P < 0.01$) than for *in vitro*-derived embryos. Embryo treatment (fresh vs. vitrified) did not significantly affect pregnancy rate. Pregnancy rate for ewes detected (by vasectomized rams) in estrus within 48 h of progesterone pessary removal was higher ($P < 0.05$) than for both the 48–68 h and unmarked groups. The latter two groups did not differ significantly. None of the first-order interactions were significant ($P > 0.05$). This study demonstrates that ovine embryos (*in vitro* and *in vivo*) can be vitrified, thawed, and transferred without compromising embryo viability. However, the differences in pregnancy rate between the recipient groups warrant further investigation. The MVC cryotop method is a vitrification technique that can be adapted to routine field use.

Table 1. Pregnancy rate of fresh and vitrified *in vivo* and *in vitro* ovine embryos after embryo transfer

Embryo source	Onset of estrus	Ewes pregnant (%)	
		Fresh embryos	Vitrified embryos
<i>In vivo</i>	<48 hour	9/11 (81.8)	9/11 (81.8)
	48–68 hour	10/18 (55.6)	12/19 (63.2)
	Unmarked	8/15 (53.3)	3/12 (25.0)
<i>In vitro</i>	<48 hour	9/13 (69.2)	8/14 (57.1)
	48–68 hour	3/14 (21.4)	6/16 (37.5)
	Unmarked	2/10 (20.0)	4/12 (33.3)

89 EVALUATION OF A CUSHIONED CENTRIFUGATION TECHNIQUE FOR PROCESSING BOAR SEMEN FOR FREEZING

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Boar semen freezing procedures include the use of centrifugation to concentrate sperm and remove seminal plasma prior to dilution in freezing extender. The centrifugation techniques employed have necessarily been a compromise between the need to recover as many spermatozoa as possible after centrifugation and the damage caused by pelleting the sperm. The use of an inert, dense, and isotonic solution as a cushion in the bottom of the tube leads to the use of higher-speed centrifugation to ensure maximum sperm recovery. However, it is necessary to know the viability and functionality of the samples after the thawing process. The aim of this work was to evaluate the effect of cushion-technique centrifugation on the *in vitro* sperm viability and the penetrating capacity after thawing. Sperm-rich fractions from five fertile boars were diluted and cooled to 15°C before centrifugation. Two centrifugation regimes were used: 800g for 10 min called the "standard method" (SM) (Westendorf P *et al.* 1975 Dtsch. Tierärztl Wochenschr. 82, 261–267) and 1000g for 20 min on an iodixanol isotonic solution 60% w/v gradient (Sigma Chemical Co., St. Louis, MO, USA) called the "cushion method" (CM). Spermatozoa were diluted in lactose/egg-yolk extender, cooled to 5°C over 2 h and then frozen with glycerol and Equex by classic methodology (Westendorf P *et al.* 1975 Dtsch. Tierärztl Wochenschr. 82, 261–267). Frozen sperm samples were thawed in a circulating water bath at 38°C for 30 s. To detect increases in plasma membrane lipid packing disorder and viability, frozen-thawed samples of sperm were stained with merocyanine 540 (M540) and Yo-Pro 1 (Harrison *et al.* 1996 Mol. Rep. Dev. 45, 378–391) and evaluated by flow cytometry. *In vitro* penetration ability was assessed using the homologous *in vitro* penetration (hIVP) test with immature oocytes (Gadea and Matas 2000 Theriogenology 54, 1343–1357). ANOVA analysis revealed that centrifugation by CM showed higher values of intact viable spermatozoa than SM centrifugation (60.21 v. 54.68%, $P < 0.05$). The *in vitro* penetration assay showed no differences in penetration rate or mean number of sperm penetrated per oocyte. However, significant boar and interaction effects were found ($P < 0.01$). These results indicated that different effects of the treatment were found for every boar. In conclusion, the cushioned centrifugation method gives a simple means of processing porcine semen for freezing more efficiently without loss of fertilizing capacity.

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90 PRESERVATION OF HERITAGE LIVESTOCK BREEDS: INTEGRATED PROGRAM TO CRYOPRESERVE GERMLASM FROM TENNESSEE MYOTONIC GOATS

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The genetic diversity of common commercial livestock has diminished precipitously due to intensive selection by agribusiness. Rare and/or heritage breeds of domestic livestock are thought to be of low commercial value, yet such breeds contain unique genes that impart valuable traits including disease and parasite resistance, efficient feed conversion, high fecundity, and unique food and fiber qualities. These irreplaceable genetics can be preserved by the establishment of a comprehensive germplasm preservation program whereby the complete genetics of a given unimproved breed can be reestablished in a single generation as needed. We describe and demonstrate here the successful preservation of germplasm from the Tennessee Myotonic (TM) goat breed, and the resulting initiation of a comprehensive germplasm cryolibrary of heritage livestock breeds. Cycling mature TM does ($n = 18$) were synchronized (PGF2 α , 7.5–10 mg i.m.), superovulated (FSH, 50–40–30 mg bid i.m., decreasing over 3 days), and bred by natural service. Embryos were collected surgically from the uterus on Day 7 and good-excellent grade embryos were cryopreserved using a conventional multi-step freezing protocol and stored in LN₂. Semen from mature TM bucks ($n = 15$) was collected biweekly (AV and teaser) and processed using a conventional slow cooling/LN₂ plunge protocol. For each animal, a primary cell culture, derived from skin biopsy, was propagated (3 passages) and frozen, and blood (serum, plasma) was collected and cryobanked. A total of 242 embryos, representing 25 sire/dam pedigrees, and 1140 straws of semen were cryobanked. Embryo viability was confirmed by surgical transfer of thawed embryos (2 embryos into each of 2 does) resulting in the birth of 1 healthy buck. Semen viability was assessed by microscopy (30–50% motility; 10–55% live by fluorescence staining) and by AI with frozen-thawed semen (pregnancies confirmed by ultrasound). This report documents and validates the utility of contemporaneous cryopreservation methods to successfully establish a cryolibrary of germplasm from rare breeds of livestock. This strategy affords long term safe storage of viable germplasm with the capability for rapid and accurate reestablishment of the breed genetics if/when needed. Moreover, such storage provides material for assessment of quantitative genetic diversity and for testing of breed disease susceptibility/resistance should that be warranted in the future.

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91 EMBRYO DEVELOPMENT AFTER ICSI OF EQUINE OOCYTES VITRIFIED BEFORE AND AFTER IVM

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Vitrification has proven to be the method of choice for cryopreservation of mammalian oocytes. In this study, we evaluated *in vitro* embryonic developmental competence of equine oocytes, vitrified before and after IVM, and fertilized by ICSI. The benefits of the interaction between

Naloxone (Nx) and endogenous opioids peptide receptors in different conditions of cellular stress have already been demonstrated (Sheu *et al.* 1997 *Biochem. Biophys. Res. Comm.* 231, 12–16). In this study we determined whether addition of Nx to the vitrification solutions can limit the oocyte's damages. COCs collected April to June from abattoir ovaries were: (1) vitrified immediately after recovery (PREM) or (2) matured for 24 h in TCM 199 (Galli *et al.* 2002 *Theriogenology* 58, 705–708) before vitrification (POSTM). Half of the oocytes of the two groups were vitrified using solutions supplemented with 10^{-8} M Nx. Cryoprotectants were loaded in three steps as reported by Maclellan *et al.* (2002 *Theriogenology* 58, 911–919). Oocytes were placed on a nylon cryoloop (Hampton Research, Laguna Niguel, CA, USA) and immediately plunged into liquid nitrogen. Oocytes were thawed by immersing the loop sequentially in 0.25 M, 0.188 M, and 0.125 M sucrose in HEPES synthetic oviductal fluid (HSOF) for 30 s per step. PREM oocytes were subjected to 24 h IVM, POSTM were cultured 2–3 h after thawing. Matured oocytes, as assessed by the presence of the first polar body, underwent ICSI. Frozen semen was separated over a discontinuous Percoll gradient and denuded oocytes were injected with a single spermatozoon. Non-vitrified oocytes matured under the same conditions were used as a control. Injected oocytes were cultured in SOFaa until Day 9 (Day 0 day of ICSI). Vitrification was done in five replicates and all oocytes were injected on the same day. Chi-square test was used for statistical analysis (Statistica for Windows; Stat Soft, Inc., Tulsa, OK, USA); significance was assessed at $P < 0.05$. Results are reported in Table 1. The number of degenerated oocytes and the cleavage rates were not significantly different among treatments ($P > 0.05$). Within vitrified COCs, only those with Nx in the vitrification solutions reached the blastocyst stage at Day 9; because of the low number of oocytes used in this work, blastocyst rate was not different among treatments. Further studies are needed to evaluate the benefits of adding Nx to oocyte vitrification solutions.

Table 1. Embryo development after ICSI of vitrified equine oocytes

		Recovered	Degenerated	Injected	Cleaved (%)	Blast D 9
PREM	Nx	12	6	4	2 (50.0)	1
	–	21	6	9	5 (55.5)	0
POSTM	Nx	35	15	11	5 (45.5)	1
	–	29	10	10	5 (50.0)	0
Control		60	12	23	17 (74.0)	7

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92 BLASTOCYST FORMATION FROM VITRIFIED BOVINE OOCYTES, ZYGOTES, AND TWO-CELL EMBRYOS

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The objective of this study was to devise a protocol to preserve bovine oocytes and early cleavage-stage embryos by vitrification and to compare their subsequent embryonic development after *in vitro* fertilization (IVF). Mature bovine oocytes from a commercial source (BoMed; Madison, WI, USA) were randomly allocated (in four replicates) to four treatment groups. Group I: control oocytes were subjected to IVF and cultured in CR1aa medium in a humidified atmosphere of 5% O₂/5% CO₂/90% N₂ at 38°C. Group II: MII-stage oocytes were subjected to vitrification and then fertilized by IVF. Group III: presumptive zygotes were vitrified after IVF. Group IV: two-cell embryos resulting from IVF that were cultured for ~28 h before vitrification. The vitrification solution consisted of TCM199 medium supplemented with 10% fetal bovine serum (mTCM) and containing 20% ethylene glycol (EG)/20% dimethyl sulfoxide (DMSO)/0.65 M trehalose. The oocytes/embryos to be vitrified were rinsed in mTCM, then in 5% EG/5% DMSO, then in 10% EG/10% DMSO, and finally for 45 s in the vitrification solution. For vitrification, groups of 6 to 12 oocytes/embryos were pipetted in <1-μL volume of vitrification medium onto the tip of a CryoTop (Katayama *et al.* 2003 *Fertil. Steril.* 80, 223); plunged directly into liquid nitrogen (LN₂), and stored for ~2 h. Vitrified samples were warmed and liquefied by rapidly transferring the Cryotops from LN₂ into 0.25 M trehalose in mTCM at 37°C and then sequentially at 1-min intervals into 0.188 M and 0.125 M trehalose. Cleavage was evaluated on Day 3 post-insemination, and blastocyst development was assessed on Days 7 and 9 post-insemination. Of the 251 oocytes in Group I, 71% cleaved by Day 3, 21% formed blastocysts by Day 7, and 29% did so by Day 9; 3% of the total hatched. Of the 116 oocytes in Group II, fewer cleaved ($P > 0.05$) by Day 3 (54%) and developed into blastocysts by Day 7 (4%) and by Day 9 (8%); none hatched. Group III zygotes ($n = 131$) responded like Group II oocytes, 53% cleaved, and 5% formed blastocysts on Day 7 and 7% on Day 9; none hatched. In contrast, 19% of the 122 two-cell embryos formed blastocysts by Day 7 and 28% by Day 9, and 3% hatched. Although significantly fewer oocytes/embryos in Groups II and III cleaved compared with Group I, more than 50% of them did so after vitrification. After fertilization and cleavage, the two-cell embryos were much more resistant to the deleterious effects of cryoprotectants and vitrification. Higher survival of two-cell embryos may result from their increased permeability to cryoprotectants, and to water due to their higher surface area to volume ratio.

93 BLASTOCYST PRODUCTION FROM BOVINE OOCYTES VITRIFIED IN A CLOSED (BIOSECURE) SYSTEM FOLLOWING *IN VITRO* MATURATION IN THE PRESENCE OR ABSENCE OF VITAMIN E

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The value of assisted reproductive technologies intended for conservation of livestock genetics ultimately will depend on their effectiveness in both sustaining gamete/embryo viability and ensuring stringent biosecurity. This study investigated bovine oocyte survival following vitrification

in a sealed system prior to storage in liquid nitrogen. It also tested the effect of supplementary vitamin E on tolerance of oocytes to vitrification procedures. Healthy COCs from abattoir-derived ovaries were matured in TCM-199 supplemented with 10% v/v fetal calf serum (FCS) in the absence (control) or presence (VitE) of 100 μ M α -tocopherol (Sigma, Poole, UK) in humidified atmosphere (5% CO₂ in air; 38.5°C). Between 22 and 24 h after commencement of IVM, COCs were pipetted to remove excess cumulus cells, and then equilibrated at room temperature in 7.5% DMSO plus 7.5% ethylene glycol (EG) in HEPES-buffered Holding Medium (HM; Irvine Scientific, Santa Ana, CA, USA) for 7 min before transfer to vitrification solution (15% DMSO, 15% EG, and 0.5 M sucrose in HM; Irvine Scientific). Loading of oocytes ($n = 78$ control and 85 VitE) into CryoTips (Irvine Scientific) and heat-sealing (each end) was achieved within 90 s, with tips then plunged into liquid nitrogen. Subsequent warming and cryoprotectant removal were at room temperature in HM with 1 M sucrose for 2 min, 0.5 M sucrose for 4 min, and HM alone for 6 min. Oocytes were allowed to recover for approximately 3 h in TCM-199 with 20% FCS (5% CO₂ in air; 38.5°C), and then fertilized *in vitro* (single sire). After 22 h (Day 1) presumptive zygotes were transferred to SOF containing fatty acid-free BSA (4 mg mL⁻¹) and incubated for up to 8 days (5% O₂, 5% CO₂, 90% N₂; 38.5°C). Cleavage data (Day 2) and blastocyst yields (Days 7 to 9) were analyzed by chi-square test. In addition to those that were vitrified, some oocytes ($n = 9$ per treatment) were observed via video to permit analysis (ImageJ; NIH, USA) of volume excursions during the 7 min immediately following initial exposure to HM with 7.5% DMSO plus 7.5% EG. Data were compared using ANOVA. Overall incidence of cleavage by Day 2 was 45% (range: 36 to 51%) and 35% (31 to 43%) for control and VitE, respectively, (NS). Day 7 and total control blastocyst yields were 7.4% and 18.5%, respectively; corresponding yields for VitE were 19% and 25% (control vs. VitE, NS). Video evidence indicated that although Control oocytes invariably reached minimal volume later than VitE oocytes (30 vs. 20 s), in each case this was 52% of initial size. By 7 min, both had similar volumes, the respective means being 94% and 92% of initial size. In the present study provision of vitamin E during IVM did not significantly enhance the subsequent resilience and development of oocytes subjected to a vitrification protocol. However, this protocol achieved efficient and biosafe bovine gamete storage.

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94 COMPARING DIFFERENT LEVELS OF OSMOLARITY AND pH OF LACTOSE EXTENDER ON THE VIABILITY OF SPERMATOZOA IN THE BACTRIAN CAMEL (*CAMELUS BACTRIANUS*)

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Lactose has been used widely as a semen extender for camels although in the absence of evidence illustrating its suitability. Considering the osmolality (316.1 ± 1.48 mOsm/kg) and pH (7.4 ± 0.03) of Bactrian camel semen (Mosaferi S *et al.* 2004 Theriogenology, in press), the objective of this study was to investigate the effect of osmolality and pH of lactose extender on the viability of Bactrian camel spermatozoa. In Experiment I, with pH adjusted to 6.9, the effect of lactose concentrations of 9, 10, 11, 12, and 13% with an osmolality of 290, 333, 350, 376, and 419 mOsm/kg, respectively, on the viability of spermatozoa was investigated. In Experiment II, with lactose fixed at 10%, the effect of extender with pH of 5.9, 6.9, 7.5, 7.9, and 8.9 on the viability of spermatozoa was examined. All extenders contained 20% egg yolk. In both experiments, semen was collected from camels with a sound history of semen quality and fertility ($n = 3$), using a modified artificial vagina, and divided into different treatments after mechanical removal of semen viscosity (Mosaferi *et al.* 2004). Progressive forward motility of spermatozoa was examined at the time of semen collection and at 4, 12, and 24 h after incubation at 4°C. Data were analyzed using the GLM procedure in SAS/STAT (SAS Institute, Inc., Cary, NC, USA) after arcsine transformation. At the time of semen dilution, the progressive forward motility of spermatozoa was greater at osmolality of 290 (28.5%), 333 (34%), and 350 (31%) compared to 376 (13.5%) and 419 (1%) mOsm/kg ($P < 0.05$). The same trend in the progressive forward motility of spermatozoa was noticed after 4 h of incubation at 4°C; although a significant decrease ($P < 0.05$) occurred at 290 (11%), 333 (18%) and 350 (16%). After 12 and 24 h of incubation at 4°C, the progressive forward motility of spermatozoa was less than 10% at 333 and 350 mOsm/kg ($P < 0.05$). At the time of semen dilution, the progressive forward motility of spermatozoa was greater ($P < 0.05$) at pH 6.9 (35.5%) and 7.5 (18%) compared to pH 5.9 (0%), 7.9 (7.5%) and 8.9 (2.5%). The same trend in the progressive forward motility of spermatozoa was observed after 4 h incubation at 4°C; although, a significant decrease ($P < 0.05$) occurred at pH 6.9 (15%) and 7.5 (9%) at this time. After 12 h incubation at 4°C, the progressive forward motility of spermatozoa was less than 5% at pH 6.9 and 7.5 ($P < 0.05$). In conclusion, 10% (333 mOsm/kg) and 11% (350 mOsm/kg) lactose, at the adjusted pH of 6.9, were the most suitable concentrations of lactose extender for preserving Bactrian camel semen for less than 4 h after which the viability of spermatozoa deteriorated significantly in this extender.

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95 EFFECT OF ESTROUS COW SERUM ON SURVIVAL OF *IN VITRO*-PRODUCED BOVINE EMBRYOS AFTER SLOW FREEZING OR VITRIFICATION

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Until now, the major obstacle associated with the extensive use of *in vitro*-produced bovine embryos is the lack of suitable methods to cryopreserve them. At least two approaches exist for overcoming this problem. One is to adjust cryopreservation methods to the requirements of these embryos,

and the other is to improve embryo quality by using an appropriate *in vitro* environment for embryo production. The objective of this study was to determine the effect of estrous cow serum (ECS) during *in vitro* culture on embryo survival after cryopreservation by slow freezing or vitrification. Cumulus-oocytes complexes were *in vitro*-matured and fertilized as previously described (Ferre *et al.* 2003 Theriogenology 59, 301 abstr). Presumptive zygotes were denuded from cumulus cells and cultured in groups of 50 in 400 μ L drops of CR1aa medium. Seventy-two hour post-insemination (PI) embryos were randomly separated into three groups. Each group was then cultured in CR1aa + 5% ECS (without BSA; CR1-ECS), CR1aa + 3 mg/mL BSA (CR1-BSA), or CR1aa + 5% ECS + 3 mg/mL BSA (CR1-ECS-BSA). Embryos were cultured under 38.5°C, 5% CO₂, 5% O₂, and 90% N₂. At 7.5 days PI, blastocysts from each group were double stained using propidium iodide and bisbenzimidazole (Hoechst 33342) to determine damaged cells and total cell number. The remaining embryos were randomly cryopreserved by freezing (1.5 M ethylene glycol; cooled at 0.5°C/min to -35°C) or vitrification (open pulled straw, Vajta *et al.* 1998 Mol. Reprod. Dev. 51, 53–58). After thawing or warming, embryos were cultured in CR1-ECS-BSA to evaluate embryo survival (hatching rate). Data were analyzed by χ^2 , ANOVA and Student's *t*-test (SAS Institute, Inc., Cary, NC, USA). Total cell number was higher in embryos cultured in CR1-ECS than in CR1-BSA or CR1-ECS-BSA (CR1-ECS: 142.1 $\pm$ 4.7, *n* = 23 vs. CR1-BSA 124.7 $\pm$ 4.9, *n* = 21, and CR1-ECS-BSA 125.8 $\pm$ 4.5, *n* = 25; *t*-test, *P* < 0.05). No differences were found in percent of damaged cells (CR1-ECS: 0.7%; CR1-BSA: 1.8%; CR1-ECS-BSA: 0.7%). Blastocyst survival after thawing was affected by cryopreservation methods and culture media (*P* < 0.01, Table 1). No interaction was found between both factors. In conclusion, under our experimental conditions elimination of ECS from CR1aa medium improves embryo cryotolerance. Vitrification allows for higher survival rates, regardless of the presence of serum during embryo culture.

Table 1. Effect of cryopreservation method and serum supplementation during embryo culture on survival rate of *in vitro*-produced bovine embryos

	Treatments	Hatched/Total (%)
Culture medium	CR1-ECS	37/172 (21.5) ^b
	CR1-BSA	73/181 (40.3) ^a
	CR1-ECS-BSA	37/187 (19.8) ^b
Cryopreservation method	Freezing	33/275 (12) ^a
	Vitrification	114/265 (43) ^b

^{a,b} Different letters within cryopreservation methods and culture media differ significantly *P* < 0.01 (χ^2).

96 A NOVEL EXTENDER FOR PRESERVATION OF BACTRIAN CAMEL (*CAMELUS BACTRIANUS*) SEMEN

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Lactose and Green buffer (IMV, France) are the most commonly used extenders for camel semen. The viability of Bactrian camel spermatozoa in lactose extender is reduced after 4 h of incubation at 4°C (unpublished data). Although Green buffer is used for dromedary camel semen, there are no data indicating its effectiveness for Bactrian camel semen. More recently, we reported that the osmolality and pH of Bactrian camel semen are 316.1 $\pm$ 1.48 mOsm/kg and 7.4 $\pm$ 0.03, respectively. The objective of this study was to compare three different semen extenders, to determine if a TRIS-based diluent (SHOTOR* Diluent), a completely defined diluent, can maintain cooled camel sperm as effectively as established diluents. SHOTOR Diluent consists of 2.6 g TRIS, 1.35 g citric acid, 1.2 g glucose, and 0.9 g fructose in 100 mL of deionized water, with an osmolality of 330 mOsm/kg and pH of 6.9. SHOTOR Diluent, lactose, 10% (w/v), with an osmolality of 330 mOsm/kg and pH of 6.9, and Green buffer were compared in this study. All extenders contained 20% egg yolk. Semen was collected from bulls with a sound history of semen quality and fertility (*n* = 3), using a modified artificial vagina, and divided equally into the different extenders (Mosaferi *S et al.* 2004 15th Int. Cong. Anim. Reprod. 2, 520; Mosaferi *S et al.* 2004 Theriogenology, in press). Progressive forward motility and percentage of live spermatozoa were examined at the time of semen collection (time 0) and after 4, 12, and 24 h of incubation at 4°C. Data were analyzed using the GLM procedure in SAS/STAT after arcsine transformation. The forward progressive motilities of spermatozoa at 0, 4, 12, and 24 h after semen collection were 65.5, 54, 44.5, and 36.5% in SHOTOR Diluent; 31, 18.5, 8.5, and 0% in 10% lactose; and 60.5, 54.5, 33, and 32.5 % in Green buffer, respectively (Table 1). The percentage of live spermatozoa at 0, 4, 12, and 24 h were 84.5, 84, 81 and 74.5% in SHOTOR Diluent; 80, 79.5, 72.5, and 56.5% in 10% lactose; 89, 82.5, 82.5, and 77.5% in Green buffer, respectively (*P* > 0.05). The progressive forward motility of spermatozoa did not significantly decrease by 12 h at 4°C in SHOTOR Diluent (*P* > 0.05; Table 1), whereas it significantly decreased after 4 h and 12 h of incubation at 4°C in Green buffer and 10% lactose, respectively (*P* < 0.05; Table 1). Further decrease in the progressive forward motility occurred in all extenders after 24 h at 4°C (*P* < 0.05; Table 1). In conclusion, SHOTOR Diluent is better than Green buffer and 10% lactose as an extender for chilled storage of Bactrian camel semen for 12 h at 4°C.

* Shotor means camel in the Persian language.

Table 1. The progressive forward motility of Bactrian camel spermatozoa extended in SHOTOR Diluent (1), 10% lactose (2) and Green buffer (3) at the time of semen collection (time 0) and after 4, 12, and 24 h of incubation at 4°C

Extender Time	0 h	4 h	12 h	24 h
1	65.5 ^{Aa}	54 ^{Aab}	44.5 ^{Aab}	36.5 ^{Ab}
2	31 ^{Ba}	18.5 ^{Bb}	8.5 ^{Bbc}	0 ^{Bc}
3	60.5 ^{Aa}	54.5 ^{Aab}	33 ^{ABb}	32.5 ^{Ab}

^{abc} Values within rows with different superscripts differ ($P < 0.05$).

^{AB} Values within columns with different superscripts differ ($P < 0.05$).

The authors wish to thank the director and station staff of Bactrian Camel Research Center at Meshkinshahr, Ardabil, for providing facilities and kind assistance throughout the experiment.

97 ASSESSMENT OF VIABILITY OF *IN VITRO* PRODUCED BOVINE EMBRYOS FOLLOWING VITRIFICATION BY CVM OR SLOW FREEZING WITH ETHYLENE GLYCOL AND TRIPLE TRANSFER

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Vitrification has become the method of choice for the preservation of *in vitro* derived embryos of a number of species, and several methods of vitrification have been developed. One such method, the cryoLogic vitrification method (CVM) yields high survival rates of warmed embryos (Lindemans W *et al.* 2004 Reprod. Fert. Dev. 16, 174 abstr). In this study, the post-warm viability of bovine IVP embryos following either vitrification using CVM or slow freezing using ethylene glycol (EG) was compared. In addition, the survival of embryos following triple transfer to synchronized recipients was measured and the embryo (“e”) and recipient (“r”) contributions to embryo survival was determined using the “er” model for embryo survival (McMillan WH *et al.* 1998 Theriogenology 50, 1053–1070). Bovine IVP methods were those of van Wagtenonk *et al.* 2004 Reprod. Fert. Dev. 16, 214 (abstr). On day 7 of culture (Day 0 = IVF), Grade 1 and 2 embryos that had reached at least the late morula stage were selected for vitrification (20% DMSO, 20% ethylene glycol) or freezing in 1.5 M ethylene glycol + 0.1 M sucrose (0.5°C/min to –35°C). Following storage in LN₂ for at least 24 h the embryos were thawed, the cryoprotectant removed, and the embryos cultured for 72 h in mSOF medium under 5% CO₂, 7% O₂, 88% N₂. The number of hatching embryos was recorded at 24-h intervals. In addition, blastocyst and expanded blastocyst embryos were thawed and immediately transferred nonsurgically to recipients (three embryos of the same grade to each recipient) on Day 7 of a synchronized cycle (Day 0 = heat). The recipients were ultrasound-scanned for the presence of, and number of, fetuses on Days 35 and 62, respectively. The *in vitro* assessment of 148 CVM and 230 EG frozen embryos indicated that more vitrified than EG embryos hatched by 72 h (73% vs. 62%; CVM vs. EG, $\chi^2 = 4.5$, $P < 0.05$). Overall, more Grade 1 embryos hatched than Grade 2 (74% vs. 60%, $\chi^2 = 7.2$, $P < 0.01$). CVM embryos (105) were triple-transferred to 35 recipients, and EG embryos (30) were triple-transferred to 10 recipients. Recipient pregnancy rates at Day 62 were 80% and 70%, respectively. Overall embryo survival was 38.5% (41% for CVM and 30% for EG). The overall calculated “e” and “r” values were 0.39 and 1.0 (“e”: 0.42 and 1.0, and “r”: 0.31 and 1.0, respectively, CVM and EG groups). Survival rates of CVM embryos to Day 62 (41%) were slightly lower than that previously obtained for fresh embryos produced using an identical IVP procedure (44% – van Wagtenonk AM 2004).

98 THE EFFECT OF HIGH HYDROSTATIC PRESSURE ON THE MOTILITY OF FRESH AND FROZEN-THAWED BULL SEMEN

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Previously, we reported that a sublethal shock, high hydrostatic pressure (HHP), significantly improves the post-thaw survival of frozen mouse blastocysts, presumably from the induction of shock proteins (Pribenszky *et al.* 2004 Reprod. Fert. Dev. 16, 181). Others reported that HSP90 in spermatozoa decreased substantially after freezing (Huang *et al.* Theriogenology 51, 1007–1016; Cao Wen-Lei *et al.* 2003 Asian J. Androl. 5, 43–46). We now report the effect of HHP on motility of the fresh bull semen to determine whether sperm survives in an altered pressure environment, and to compare post-thaw motility of HHP-treated frozen bull semen with controls. The survival rates were compared by chi-square test. Expt 1: Semen of one bull was diluted to a sperm concentration of 8×10^7 /mL with AndroMed extender (MiniTüb, Tiefenbach, Germany). Diluted sperm was loaded into 0.25-mL straws at 25°C. Each straw was cut in half. One demi-straw was heat-sealed and exposed to HHP, and sperm in the companion demi-straw served as a control. Experiments were replicated eight times for each pressure/time treatment. Progressive motility was assessed independently by light microscopic investigation by two individuals. The treatment groups were: 10 MPa for 30, 60, 90, or 120 min; 30 MPa for 30, 60, 90, 120, or 510 min; 50 MPa for 30, 60, or 90 min; 70 MPa for 30, 60, or 90 min; and 90 MPa for 30, 60, 90, 120, or 510 min. The average motility of the control samples ranged from 75 to 90%, while the average motility of the pressurized samples ranged between 55 (90 MPa/120 min) to 84% (10 MPa/30 min). The groups of 30 MPa/510 min and 90 MPa/510 min exhibited significantly lower motility compared to the other pressurized groups (27% and 33%, respectively; $P < 0.05$). Expt 2: Semen was collected from two bulls with poor sperm freezability. Semen was diluted as described

for the first experiment, loaded into straws, and assigned to one of 4 treatment groups. Half the straws from each bull were exposed to 90 MPa/30 min, 90 MPa/90 min, 30 MPa/30 min, or 30 MPa/90 min, and then cryopreserved. Controls consisted of straws that were cryopreserved without pressure treatment. Cryopreservation steps were 60 min equilibration at 5°C, followed by 10 min at -110°C, and then plunging into liquid nitrogen. Straws were thawed in a 35°C water-bath for 30 s. Each treatment and control group was replicated 8 times (8 samples per bull). The average post-thaw motility was significantly superior with pressure pre-treatment in each of the pressurized groups compared to the samples frozen without previous pressurization ($P < 0.001$) (Bull I: 2–3% without pressurization vs. 17–33% with pressurization; Bull II: 0% without pressurization vs. 21–35% with pressure pre-treatment). Among the pressure/time parameters used, 30 MPa/90 min proved significantly superior (33 and 35%; $P < 0.05$) for each of the bulls. Expt. 2 clearly demonstrates the beneficial effect of a previous pressure treatment on post-thaw motility of bull semen cryopreserved in our experiment. Further investigations are needed, including samples from different bulls, different freezing protocols, and the biological background of the process.

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99 COMPARISON OF *IN VITRO* DEVELOPMENT FOLLOWING CRYOPRESERVATION OF MEISHAN AND WHITE CROSS SWINE EMBRYOS

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Development of improved protocols for cryopreservation of zona pellucida-intact porcine embryos could greatly impact the swine industry. Our aim was to investigate *in vitro* development following cryopreservation of embryos from Chinese Meishan (M) and occidental white cross (WC) breeds using a modified protocol described previously (Misumi K *et al.* 2003 *Theriogenology* 60, 253–260). First-parity M sows ($n = 11$) and WC gilts ($n = 13$) were observed for estrus every 12 h and inseminated at 12 and 24 h after estrous onset within breed using semen from 2 different boars. Females were sacrificed between Days 4.5 and 6 after estrus and embryos were collected using Beltsville embryo culture medium (BECM). Compact morula (CM) or blastocyst stage embryos from each female within breed were randomly allocated either directly into the culture system to serve as controls (68 M and 48 WC embryos) or to undergo cryopreservation. A total of 101 M and 78 WC embryos were cryopreserved using the following protocol: (1) 5 min in BECM + 10% ethylene glycol (EG); (2) 5 min in BECM + 10% EG + 0.27 M sucrose + 1% polyethylene glycol (PEG); and (3) 30 to 45 s in BECM + 40% EG + 0.36 M sucrose + 2% PEG. In the last solution, 5 to 10 embryos in a 5- to 10- μ L microdrop attached to a fine glass pipette were exposed to the vapor phase of liquid nitrogen (LN₂) for 15 s and then plunged into LN₂. The pipette tip was broken and the tip and associated frozen microdrop were placed inside an LN₂-submerged 2-mL cryotube containing a hole in the lid for 1 h. Next, embryos were thawed using a 4-step (5 min each) procedure: (1) BECM + 5% EG + 0.57 M sucrose; (2) BECM + 2.5% EG + 0.29 M sucrose; (3) BECM + 0.3 M sucrose; and (4) BECM alone. All procedures were performed with solutions maintained at 37°C. Cryopreserved and control embryos were cultured in 50 μ L drops of modified Whitten's medium + 1.5% BSA under oil at 37°C in a 5% CO₂ in air environment and scored daily for development. For embryos undergoing cryopreservation, retrieval rates from cryovials were 92% and 96% for M and WC, respectively. The percentage of embryos surviving 24 h after cryopreservation without lysis or degeneration was higher for M (72%) than for WC (44%; $P < 0.001$; χ^2 -test). However, *in vitro* development of embryos that survived cryopreservation was not different between M and WC at the expanded (64%) or hatched (22%) blastocyst stages. Developmental rates were significantly higher for control embryos than for frozen embryos from both breeds. Rates of expanded blastocyst formation did not differ between M and WC control embryos (98% and 95%, respectively), but more M embryos developed to the hatched blastocyst stage (22% for M v. 9% for WC; $P < 0.05$). Our results suggest that M embryos have a higher capacity to survive the vitrification process than WC embryos.

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Developmental Biology

100 DIET AND FATTY ACID COMPOSITION OF BOVINE PLASMA, GRANULOSA CELLS, AND CUMULUS-OOCYTE COMPLEXES

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The effects of altering dietary carbohydrates and lipids to oocyte donors during superovulation and ovum pickup (OPU) on *in vitro* embryo production was reported previously (Adamiak *et al.* 2004 *Reprod. Dev. Fert.* 16, 193–194). Here we report the effects of these dietary treatments on the fatty acid (FA) composition of plasma, granulosa cells (GCs), and cumulus-oocyte complexes (COCs) from the 32 heifers used in that study. Blood samples were collected by jugular venipuncture. COCs and GCs were harvested from each heifer by OPU as described previously but were pooled between pairs of heifers within treatment to provide adequate material for FA analysis. Both GCs and COCs were washed twice in PBS supplemented with 0.3% (w/v) BSA (FA-free) before being transferred into 2:1 (v/v) chloroform: methanol solution for FA extraction. FA composition was determined using gas chromatography as described previously (Reis *et al.* 2002, *Theriogenology* 57, 507). Data were analyzed by ANOVA. Total plasma FA content averaged 1.12 μ g/mL and was unaffected by body condition score (BCS). Low BCS heifers had more saturated (54.0 ± 1.76 vs. $49.2 \pm 1.74\%$) and monounsaturated (22.4 ± 1.08 vs. $18.2 \pm 0.69\%$) FA, but less polyunsaturated FA (PUFA) (23.7 ± 1.75 vs. $32.8 \pm 2.21\%$) in plasma than moderate BCS heifers ($P < 0.01$). Animals fed high relative to low fiber diets had greater plasma FA (1.3 ± 0.15 vs. 1.0 ± 0.12 μ g/mL)