**Cryopreservation/Cryobiology**

**37 EFFECT OF EMBRYO STAGE ON PREGNANCY RATE FOLLOWING DIRECT TRANSFER OF BOVINE EMBRYOS FROZEN IN ETHYLENE GLYCOL**

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Annually, more than 400,000 *in vivo*-recovered bovine embryos are officially reported by members of the Canadian and American Embryo Transfer Associations. Between 65 and 70% of these embryos are cryopreserved and more than 95% are frozen in ethylene glycol (EG). Statistics on factors affecting embryo freezing are difficult to obtain because many cattle breeders/farmers no longer report pregnancy rates back to embryo transfer (ET) practitioners. Concerns are often expressed as to the optimal stage at which to freeze bovine embryos: Group 0%: 87.2% a, b, c

<table>
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<tr>
<th>No. thawed and transferred</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>Total</th>
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<tbody>
<tr>
<td>% Pregnant</td>
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|  | 45.7 | 55.3 | 52.1 | 43.8 | 54.3 |

**38 VITRIFICATION OF BOS Taurus Indicus and BOS Taurus Indicus × BOS Taurus Taurus EMBRYOS PRODUCED IN THE PRESENCE OR ABSENCE OF FETAL CALF SERUM**


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In vitro-produced *Bos taurus indicus* (zebu) and *Bos taurus indicus × Bos taurus taurus* (cross-bred) embryos behave differently when vitrified. The present experiment aimed to examine the effect of vitrification on embryos produced in the presence or absence of FCS. Cumulus-oocyte complexes (COC) were matured in TCM-199 and fertilized in human tubal fluid medium with frozen Nelore bull semen. On Day 1 (Day 0 = IVF), presumptive zygotes were cultured with SOF(aa) + BSA in the presence of FCS (Group 2.5%) or in the absence of FCS (Group 0%) until Day 7. The cleavage was analysed on Day 3 and the blastocyst rate on Day 7. Blastocysts were vitrified and, after warming (Campos-Chillón et al. 2006) the viability was evaluated. Data were analysed with ANOVA, using the general linear model (GLM) of SAS (SAS Inst Inc., Cary, NC, USA). Sources of variation in the model included FCS concentration and first-order interactions; all factors were considered fixed effects. The arcsine transformation (√y/100) was applied to percentage data. If the ANOVA was significant, means were separated using the Tukey test. There was no difference in cleavage (for zebu embryos: Group 0%: 87.2 ± 6.8; Group 2.5%: 87.4 ± 9.5; for cross-bred embryos: Group 0%: 79.6 ± 11.9; Group 2.5%: 73.1 ± 13.7; *P > 0.05*). On the other hand, zebu embryos cultured in the presence of FCS reached blastocysts at a higher rate than cross-bred embryos in the absence of FCS (for zebu embryos: Group 0%: 33.3 ± 12.4%; Group 2.5%: 46.8 ± 13.2%; for cross-bred embryos: Group 0%: 21.8 ± 8.3%; Group 2.5%: 33.6 ± 10.1%; *P < 0.05*). After vitrification and warming, no significant differences in re-expansion rate (zebu embryos: Group 0%: 82.7 ± 13.1; Group 2.5%: 75.0 ± 9.8; cross-bred embryos: Group 0%: 93.7 ± 8.8; Group 2.5%: 84.1 ± 11.3; *P > 0.05*) and cell number per embryo (zebu embryos: Group 0%: 65.1 ± 34.7; Group 2.5%: 42.6 ± 17.2; cross-bred embryos: Group 0%: 64.3 ± 44.2; Group 2.5%: 52.0 ± 31.5; *P > 0.05*) between species groups and within species were seen. However for zebu embryos, Group 0% showed a lower damaged cell ratio than Group 2.5%. The same effect was not observed in the cross-bred embryos (zebu embryos: Group 0%: 20.3 ± 22.7%; Group 2.5%: 63.3 ± 27.0%; cross-bred embryos: Group 0%: 25.4 ± 24.3%; Group 2.5%: 45.8 ± 34.6%; *P < 0.05*). The addition of 2.5% FCS had a higher deleterious effect on zebu embryos than cross-bred embryos.

Table 1. Effect of embryo stage on pregnancy rate of bovine embryos frozen in EG

<table>
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<th>Factor</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. thawed and transferred</td>
<td>37 793</td>
<td>23 012</td>
<td>10 794</td>
<td>1229</td>
<td>72 828</td>
</tr>
<tr>
<td>% Pregnant</td>
<td>54.7</td>
<td>55.3</td>
<td>52.1</td>
<td>43.8</td>
<td>54.3</td>
</tr>
</tbody>
</table>

* a,b,c *P < 0.001, chi-square.
(zebu × taurine) embryos after vitrification. These results also reinforce the species differences observed between zebu and cross-bred, as they behaved differently in relation to the addition of FCS in the culture medium and in relation to their cryopreservation sensitivity.

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39 LIPID FINGERPRINTING OF INDIVIDUAL BOVINE BLASTOCYSTS BY DESORPTION IONIZATION ELECTROSPRAY MASS SPECTROMETRY


Mass spectrometry (MS) allows the detection and structural characterisation of intact molecules such as fatty acids and complex lipids. Desorption electrospray ionization (DESI) is an ambient ionization technique used for MS analysis and profiling and imaging of drugs, metabolites and lipids directly from biological samples with no sample preparation. With the recent introduction of morphologically friendly DESI-MS solvent systems, it is also possible to acquire DESI-MS data non-destructively. Due to the extractive nature of these solvent combinations, enough ion intensity can be generated to chemical profile samples of microscopic dimensions. The objective of this work was to perform chemical profiling on intact bovine blastocysts by DESI-MS, focusing on lipid distributions. Blastocysts produced in vitro were washed 3 times in PBS + 0.1% polyvinyl alcohol to remove lipids present in the culture medium, were placed in PBS/methanol 50% and stored under –20°C for 1 week. For DESI-MS analysis, the embryos were simply placed in glass slides and allowed to dry at room temperature. Mass spectra were acquired in the negative ion mode at the mass/charge range from m/z 150 to 1000, using as solvents a combination of 1:1 (vol/vol) ethanol/dimethylformamide (DMF) or acetone/tetrile:DMF. The mass spectrometer used was a LTQ linear ion trap mass spectrometer controlled by Xcalibur 2.0 software (Thermo Fisher Scientific, San Jose, CA, USA). The lipid species detected included deprotonated free fatty acids such as palmitic acid (m/z 255.2), stearic acid (m/z 283.2), arachidonic acid (m/z 311.2) and docosanonic acid (m/z 339.3). Free fatty acid dimers appear in the region from m/z 500 to 650 and complex lipids represented mainly by glycerophospholipid classes appear in the region from m/z 700 to 1000 and include phosphatidylinositol (PI 38:1; m/z 788.7), phosphatidylserines (PS 36:1; m/z 885.7) and also the chlorinated phosphatidylcholines (PC 36:1; m/z 794.7). After recording the mass spectra, embryos could still be observed in the glass slide with evident dehydroxylation due to the action of the organic solvent. Since lipid composition of bovine embryos is closely related to cryosensitivity and due to the limited amount of analytes (each embryo is estimated to have a mass of 15 pg of total lipids) lipid analysis usually involves the pooling of individuals to have a large enough amount of analytes. Traditionally, gas chromatography is used for fatty acid residue analysis in oocytes and embryos pooled are submitted to lipid extraction and derivatization. Mass spectrometry by DESI, however, allows direct analysis of intact and single embryos and the profiling of not only free fatty acids but also complex lipids, represented mainly by 3 glycerophospholipid classes (PC, PI and PS). We envisage that DESI-MS will likely become a routine tool for the analysis of lipid composition in mammalian embryos and will contribute significantly to the development of culture systems that produce embryos with higher cryoresistance.

Support from the Purdue University Center for Cancer Research Small Grants Program is gratefully acknowledged.

40 IN VITRO SURVIVAL RATES OF IN VIVO- AND IN VITRO-PRODUCED BOVINE EMBRYOS CRYOPRESERVED BY SLOW CONTROLLED FREEZING OR VITRIFICATION

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Although slow programmable freezing is currently the standard method for bovine embryo cryopreservation, vitrification has become an alternative for in vitro-produced embryos. A study was designed to compare the in vitro survival rates of in vivo- and in vitro-produced bovine embryos with 1 of 2 commercially available methods of cryopreservation: slow freezing and solid surface vitrification. In vivo-produced Grade 1 blastocysts (n = 210) collected from superfused donor cows 7 days post-insemination and in vitro-produced Grade 1 blastocysts (n = 122) from slaughterhouse oocytes, produced with the procedure described by Chaulal et al. (2007 Theriogenology 67, 719–728) were randomly allocated in 2 groups. Group 1 (slow freezing) embryos were exposed to 1.5 M ethylene glycol (ViGro EG; Bioniche Animal Health USA Inc., Pullman, WA, USA) for 5 min and loaded in 0.25-mL plastic straws. The straws were placed in a Freeze Control CL 5500 freezer (CryoLogic, Victoria, Australia) at -80°C for 1 week. For DESI-MS analysis, the embryos were simply placed in glass slides and allowed to dry at room temperature. Mass spectra were acquired in the negative ion mode at the mass/charge range from m/z 150 to 1000, using as solvents a combination of 1:1 (vol/vol) ethanol/dimethylformamide (DMF) or acetone/tetrile:DMF. The mass spectrometer used was a LTQ linear ion trap mass spectrometer controlled by Xcalibur 2.0 software (Thermo Fisher Scientific, San Jose, CA, USA). The lipid species detected included deprotonated free fatty acids such as palmitic acid (m/z 255.2), stearic acid (m/z 283.2), arachidonic acid (m/z 311.2) and docosanonic acid (m/z 339.3). Free fatty acid dimers appear in the region from m/z 500 to 650 and complex lipids represented mainly by glycerophospholipid classes appear in the region from m/z 700 to 1000 and include phosphatidylinositol (PI 38:1; m/z 788.7), phosphatidylserines (PS 36:1; m/z 885.7) and also the chlorinated phosphatidylcholines (PC 36:1; m/z 794.7). After recording the mass spectra, embryos could still be observed in the glass slide with evident dehydroxylation due to the action of the organic solvent. Since lipid composition of bovine embryos is closely related to cryosensitivity and due to the limited amount of analytes (each embryo is estimated to have a mass of 15 pg of total lipids) lipid analysis usually involves the pooling of individuals to have a large enough amount of analytes. Traditionally, gas chromatography is used for fatty acid residue analysis in oocytes and embryos pooled are submitted to lipid extraction and derivatization. Mass spectrometry by DESI, however, allows direct analysis of intact and single embryos and the profiling of not only free fatty acids but also complex lipids, represented mainly by 3 glycerophospholipid classes (PC, PI and PS). We envisage that DESI-MS will likely become a routine tool for the analysis of lipid composition in mammalian embryos and will contribute significantly to the development of culture systems that produce embryos with higher cryoresistance.

Support from the Purdue University Center for Cancer Research Small Grants Program is gratefully acknowledged.
cryopreserved by the 2 systems, hatching rates tended to be lower \( (P = 0.01) \) with in vivo-produced embryos that were vitrified compared with slow freezing (GH/AA0, GA% vs HA/AA0, HA%). In conclusion, solid surface vitrification improved the cryo survival rates of in vitro-produced embryos compared with the conventional slow, controlled freezing procedure.

41 THE EFFECT OF AMBIENT TEMPERATURE ON SPERM MOTILITY DURING LIQUID STORAGE OF VENDA COCK SEMEN AND INDIVIDUAL DIFFERENCES IN SPERM CRYOTOLERANCE

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Improving techniques for liquid storage of cock semen can increase the efficiency of AI programs in the poultry industry. The aims of the present study were (1) to compare storage of cock sperm for 24 h at 5 and 25°C and (2) to test the cryotolerance of sperm cell motility in individual Venda cocks. Semen was collected with the abdominal massage method, from 6 indigenous Venda cocks. Cocks were 26 weeks of age and were kept under the same conditions. After macroscopic analysis, semen was pooled and diluted (1:2) with Kobidil extender and divided into 3 equal parts. Part 1 was evaluated immediately (0 h), part 2 was stored at 5°C and part 3 was stored at 25°C and evaluated for sperm motility and velocity parameters at 4, 8, 12 and 24 h of storage. For cryopreservation, semen was diluted (1:2) with modified Kobidil extender supplemented with 8% of dimethyl sulfoxide. Individual ejaculates were equilibrated at 5°C for 4 h and then loaded into the programmable freezer. Then, semen straws were thawed at 5°C. Sperm motility and velocity parameters were evaluated using the Sperm Class Analyzer\textsuperscript{B} system. Six replicates were done per trial. Data were analysed using the statistical programme GenStat\textsuperscript{B}. Treatment means were separated using Fisher’s protected t-test least significant difference \( (P < 0.05) \). Total sperm cell motility rate was 87.5% and decreased significantly during in vitro storage and was < 31% after 24 h at 25°C. Semen samples stored at 5°C showed a total sperm cell motility rate of above 50% after 24 h. There was a slight linear decrease in the percentage of sperm with progressive motility and rapid velocity as the storage period increased, irrespective of the storage temperature. The rapid and medium motility percentages were higher in fresh semen and significantly decreased \( (P < 0.05) \) during the incubation period. There was variation in the total sperm cell motility of fresh and frozen semen among cocks. There was no significant difference in variation in non-progressive and medium percentage \( (P > 0.05) \) motility in diluted fresh or frozen sperm cells or in the percentage of sperm with rapid motility in thawed semen. There was variation in <25% of the cocks in total sperm motility rate. In summary, cryopreservation reduced sperm cell motility and velocity rates in all the cock semen donors. We found that cryotolerance of cock sperm does vary among males. Furthermore, the lower temperature 5°C was suitable for semen storage of Venda cocks. This temperature \( (5°C) \) could potentially improve methods of semen equilibration before cryopreservation.

The study was supported by an Agricultural Research Council Parliamentary Grant, Department of Agriculture Forestry and Fisheries and National Research Foundation-GUN No RT21 and 24000 (NRF). The Germplasm Conservation & Reproduction Biotechnologies (GCRB) group is thanked for their support.

42 COMPARISON OF COOLING RATES IN OOCYTE VITRIFICATION SYSTEMS USING A NUMERICAL SIMULATION

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\textsuperscript{B}Facultad de Ingeniería, Universidad Nacional de La Plata and Centro de Investigación y Desarrollo en Criotecnología de Alimentos (CIDCA-CONICET), 1900, La Plata, Buenos Aires, Argentina

Interest in oocyte cryopreservation has increased due to the application of assisted reproductive technologies and the need for the establishment of ova/gene banks worldwide. In order to maintain cell viability, biological functions must be halted, inducing a suspended animation state by cooling it into a solid phase. Compared to cryopreservation of male gametes, oocytes represent a greater challenge due to their low surface area:volume. Vitrification, the solidification into an amorphous, glassy state while maintaining absence of intra- and extracellular ice crystals, requires high concentrations of cryoprotectants and extremely rapid cooling rates. Several vitrification devices such as open pulled straws (OPS), ultra fine pipette tips, nylon loops and polyethylene films have been introduced to manipulate minimal volumes and achieve high cooling rates. However, experimental comparison of cooling rates presents difficulties mainly because of the reduced size of these systems. To circumvent this limitation, a numerical simulation of cooling rates of various vitrification systems immersed in liquid nitrogen was conducted, solving the non-stationary heat transfer partial differential equation using the finite element method. Three external heat transfer coefficients \( (h = 200, 1000 \text{ and } 2000 \text{ W m}^{-2} \text{K}) \) were considered. The Cryotip\textsuperscript{B} and OPS were approached as 2 concentric finite cylinders; differential equations representing heat transfer in cylindrical coordinates were described considering radial and axial coordinates and were numerically solved as a 1-dimensional heat conduction problem in an infinite cylinder. The Cryoloop\textsuperscript{B} was approximated as a 1-dimensional heat flow system in Cartesian coordinates and Cryotop\textsuperscript{B} was numerically described as an irregular bi-dimensional axially-symmetric problem. All differential equations were numerically solved using the finite element method in COMSOL Multiphysics 3.4. The domain was discretized in triangular (Cryotip\textsuperscript{B}, OPS and Cryotop\textsuperscript{B}) and linear elements (Cryoloop\textsuperscript{B}) in order to obtain accurate numerical approximations. In each case, the warmest point of the system was identified to determine the time-temperature curve that allows the evaluation of the lowest cooling rate (worst condition). Results indicate the nylon loop (Cryoloop\textsuperscript{B}) is the most efficient heat transfer system analysed, with a predicted cooling rate of 180000°C min\(^{-1}\) for an external heat transfer coefficient \( h = 1000 \text{ W m}^{-2} \text{K} \) when cooling from 20 to –130°C; in contrast, the pipette tips (Miniflex\textsuperscript{B}) showed the lowest performance with a cooling rate of 6164°C min\(^{-1}\) at same
value of external heat transfer coefficient. Predicted cooling rates of OPS and Cryotop® (polyethylene film) were 40.909 and 37.500°C min⁻¹, respectively for the same heat transfer coefficient. It can be concluded that in oocyte cryopreservation systems, in which experimental comparison of cooling rates presents difficulties due to the reduced size of the vitrification devices, the numerical simulations and the analysis of the predicted thermal histories could contribute to determine the performance of the different techniques.

43 EFFECT OF VITRIFICATION AT GERMINAL VESICLE STAGE ON THE MITOCHONDRIAL AND CYTOSKELETAL INTEGRITY IN BOVINE OOCYTES

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In the previous report, we demonstrated that bovine germinal vesicle (GV) stage oocytes vitrified using a nylon mesh holder showed an in vitro maturation rate to the metaphase II (MII) stage similar to that of fresh ones. However, cleavage and developmental rates of vitrified oocytes were low. Because mitochondria and the cytoskeleton are thought to have a central role in energy supply and cellular division in mammalian embryogenesis, it seems possible that alternation in their function in vitrified GV oocytes may contribute to subsequent lower cleavage and developmental rates. The oxygen consumption rate reflects the mitochondrial activity and its measurement may be an effective way for non-invasive evaluation of oocyte quality. In this study, to ascertain that altered mitochondrial functions and cytoskeleton may contribute to reduce the quality of oocytes after vitrification, we evaluated the distribution of active mitochondria and the cytoskeleton in vitrified oocytes. We also examined the relationship between oxygen consumption rate and the distribution of active mitochondria in vitrified oocytes. Bovine GV oocytes connected with cumulus cells were exposed to the cryoprotectant (EFS40) in a stepwise way and transferred onto a nylon mesh holder, followed by plunging them directly into liquid nitrogen. After warming, vitrified oocytes were allowed in vitro maturation. After denuding, matured oocytes were stained with a mitochondria-specific probe, rhodamine-123 and then oxygen consumption rate using an embryo respirometer (HV-403; Research Institute for Functional Peptides, Yamagata, Japan) was measured in each oocyte. According to morphological distribution of mitochondria, oocytes were classified as follows: type 1, uniform distribution; type 2, spotted distribution; and type 3, a weak fluorescence. The oxygen consumption rate of the fresh oocytes at the MII stage was significantly (P < 0.05) higher than that of vitrified oocytes (5.29 and 4 × 10⁻⁵ mol⁻¹ s⁻¹, respectively), although there was no difference between the fresh and vitrified groups at the GV stage (5.02 and 5.06 × 10⁻⁵ mol⁻¹ s⁻¹, respectively). The oxygen consumption rates of type 1 oocytes in fresh and vitrified groups at the MII stage tended to be higher than those of type 2 and 3 oocytes (type 1, 5.29 and 5.27; type 2, 4.99 and 4.52; type 3, 4.77 and 4.48 × 10⁻⁵ mol⁻¹ s⁻¹, respectively). In addition, the percentage of type 1 oocytes in the fresh group was significantly (P < 0.05) higher than that in the vitrified group (59.4 and 34.3%, respectively). The matured oocytes also were stained with α-tubulin monoclonal antibody or F-phalloidin independently to examine the morphological status of microtubules or microfilament. The rates of oocytes with abnormal microtubules and microfilaments in the vitrified group were 29.7 and 43.5%, respectively, showing higher rates compared with corresponding fresh oocytes (9.8%; P < 0.05 and 25.0%; P = 0.21, respectively). These results suggested that the reduction of quality and subsequent developmental competence in vitrified oocytes might be related to damages of mitochondria and cytoskeleton.

44 REACTIVE OXYGEN SPECIES IN VITRIFIED BOVINE IN VITRO-MATURED OOCYTES

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Vitrification of in vitro-matured oocytes has important applications in fertility preservation and management of genetic resources. However, despite the increasing interest, the efficiency of oocyte vitrification needs to be improved. It was demonstrated that under stressful conditions cryopreserving pig oocytes accumulate reactive oxygen species (ROS; Gupta et al. 2010 Fertility and Sterility 93, 2602–2607). Reactive oxygen species are known to exert harmful effects such as mitochondrial damage, ATP (ATP) depletion, altered calcium oscillation during fertilization and consequently their developmental ability may be compromised (Takahashi et al. 2003 Mol. Reprod. Dev. 66, 143–152). The aim of the present study was to evaluate whether the exposure to cryoprotectants and vitrification procedure affect ROS production in bovine in vitro-matured oocytes. Abattoir-derived bovine (n = 360, over 6 replicates) cumulus oocyte complexes (COCs), were in vitro-matured. COCs were mechanically stripped of their cumulus cells by gentle pipetting, washed and divided into 3 groups: control (C; i.e. fresh non treated oocytes), toxicity (T) and vitrification (V) groups. In group V, oocytes were exposed to 10% ethylene glycol (EG) + 10% DMSO for 3 min, then to 20% EG + 20% DMSO and 0.5 M sucrose, loaded on cryotops and plunged into liquid nitrogen within 25 s. Oocytes were warmed into a 1.25 M sucrose solution for 1 min and then to decreasing concentrations of sucrose (0.625 M, 0.42 M and 0.31 M) for 30 s each. In group T, oocytes were simply exposed to the vitrification and warming solutions. ROS determination was carried out by a spectrofluorometer at 495 nm excitation and 525 nm emission. Frozen oocytes were thawed and incubated in 500 μL of TRIS-HCl 40 mM, pH 7.0 in the presence of 5 μmol L⁻¹ of 2',7'-dichlorofluorescein-diacetate, for 20 min at 37°C into a shaker. After incubation, the extraction was obtained by a syringe and the samples were centrifuged at 3000 rpm for 10 min at 4°C. Data were expressed as arbitrary ROS units per oocyte per min (U) and analysed by ANOVA. The results of this study showed that in bovine oocytes ROS levels tend to increase in the T and V groups compared to group C (76.0 ± 6.4, 249.9 ± 87.3 and 147.6 ± 42.6 in C, T and V groups, respectively). However, there were no statistical differences among groups and this was mainly due to the high variability recorded in both treated groups. In conclusion, these results suggest that both exposure to cryoprotectants and vitrification of in vitro-matured oocytes may influence ROS generation. However, the high variability recorded among replicates recommends further investigations.
Vitrification is a rapid freezing method in which cells/tissues are frozen in a glass state without ice crystal formation. However, vitrification of bovine oocytes is challenging due to their complex structure and sensitivity to chilling. Oocytes at the germinal vesicle (GV) stage of maturation are thought to be less prone to chromosomal and microtubular damage during cryopreservation because no spindle is present and genetic material is contained within the nucleus. However, immature oocytes are thought to be more sensitive to osmotic stress and have lower cell membrane stability than mature, metaphase II (MII) stage oocytes. The present studies aimed to validate the in vitro culture system used in our laboratory and to evaluate the effect of vitrification of bovine cumulus-oocyte complexes (COC) at different meiotic stages on their in vitro maturation (IVM), cleavage and early embryo development. Analyses were conducted on each dataset with PROC GLIMMIX in SAS using binary distribution (for yes/no response variable) and considering replicate as a random factor. In Experiment 1, meiotic progression of oocytes was evaluated at different time intervals during IVM. The following COC stages were predominantly found at different IVM time intervals: GV (90%) at 0 h, GV (47%) and germinal vesicle breakdown (GVBD; 44%) at 6 h, metaphase I (MI; 90%) at 12 h and MII (84%) at 22 h (n > 62 oocytes at each time group). In Experiment 2, bovine COC at 0, 6, 12 and 22 h of IVM were exposed to vitrification solution (15% dimethyl sulfoxide + 15% ethylene glycol + 0.5 M sucrose + 20% CS in TCM-199), loaded onto a cryotop device and vitrified by plunging in liquid nitrogen. Following warming (1 min in 0.5 M sucrose + 20% CS in TCM-199), COC completed 22 h of IVM and the nuclear stage was evaluated with lamin A/C-4′-diamidino-2-phenylindole staining. Upon completion of 22 h of IVM, 23, 23, 35 and 89% of oocytes from 0-, 6-, 12- and 22-h groups, respectively were detected at MII (P < 0.0001). In Experiment 3, cleavage and embryo development of oocytes vitrified at 0, 12 and 22 h of IVM were evaluated. The cleavage rate did not differ among vitrification groups (i.e. 14% at 0 h, 17% at 12 h and 14% at 22 h; P = 0.825). Cleavage and blastocyst rates were higher (P < 0.0001) in the non-vitrified (control) group than in vitrified groups (i.e. 73 vs 15% and 22 vs 0.3%, respectively). In conclusion, the maturation kinetics validated our in vitro culture system and vitrification adversely affected the ability of bovine oocytes to undergo in vitro maturation to the MII stage, in vitro fertilization and early embryo development. Vitrification of oocytes at GV, MI and MII stages of nuclear maturation did not differ in their subsequent survivability.

This study was supported by the Canadian Animal Genetic Resources Program, Agriculture and Agri-Food Canada.

46 PLASMINOGEN ACTIVATOR ACTIVITY IN BUFFALO IN VITRO MATURED OOCYTES AFTER VITRIFICATION-WARMING


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Plasminogen activators (PA) are proteolytic enzymes that convert plasminogen into plasmin. Plasmin is involved in physiological processes such as ovulation (Liu 2004 Front. Biosci. 9, 3356–3373), cumulus cell layer expansion (Liu et al. 1986 Endocrinology 119, 1578–1587), oocyte maturation (Dow et al. 2002 Biol. Reprod. 66, 1413–1421) and fertilization (Huarte et al. 1993 Dev. Biol. 157, 539–546). Although the interest in increasing, buffalo oocyte cryopreservation is still inefficient, especially in terms of blastocyst development after IVF. The aim of the present study was to evaluate whether exposure to cryoprotectants and the vitrification procedure affect plasminogen activator activity (PAA) in buffalo in vitro-matured oocytes. A total number of 300 cumulus-oocyte complexes over 5 replicates were selected and in vitro-matured. Cumulus-oocyte complexes were mechanically stripped of their cumulus cells by gentle pipetting, washed and divided into 3 groups (20 oocytes/group, over 5 replicates). The control group consisted of fresh in vitro-matured oocytes. In the vitrification group, denuded oocytes were first exposed to 10% ethylene glycol (EG) + 10% dimethyl sulfoxide (DMSO) for 3 min, then to 20% EG + 20% DMSO and 0.5 M sucrose, loaded on cryotops and plunged into liquid nitrogen within 25 s. Subsequently, oocytes were warmed in a 1.25 M sucrose solution for 1 min and then in decreasing concentrations of sucrose (0.625 M, 0.42 M and 0.31 M) for 30 s each. In order to test cryoprotectant effects, oocytes were simply exposed to the vitrification and warming solutions (toxicity group). Surviving oocytes were extracted by a fine needle, centrifuged at 4000 rpm for 10 min and the supernatant was mixed with the reaction solution: TRIS-HCl 0.1 M, homologous plasminogen, the chromogenic substrate for plasmin S-2251 and incubated at 37°C for 30 min. The PAA levels were measured by a spectrophotometer (405 nm) expressed as Abs/20 oocytes. The data were analysed by the Kruskal-Wallis nonparametric test. Low levels of PAA were detected in the denuded oocytes of the control, toxicity and vitrification groups. No significant differences in mean PAA values were observed among the 3 experimental groups (0.017 ± 0.001, 0.018 ± 0.002 and 0.017 ± 0.001 units, in the control, toxicity and vitrification groups, respectively). In conclusion, cryoprotectants and the vitrification procedure do not affect the proteolytic activity linked to plasmin in in vitro-matured buffalo oocytes. The results show that the vitrification/warming procedure does not exert an effect on in vitro-matured buffalo oocytes in terms of PAA generation, a parameter that plays an important role in fertilization and in vitro embryo development. Further studies are needed to identify factors affecting the efficiency of oocyte cryopreservation.
47 ROLE OF INSULIN-LIKE GROWTH FACTOR-I ON THE FUNCTIONAL PARAMETERS AND FERTILITY OF OVINE FROZEN-THAWED SEMEN


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The objective of the present experiment was to evaluate the effects of insulin-like growth factor-I (IGF-I) on the viability and fertility of ovine frozen/thawed semen. For evaluating the semen parameters, 5 rams were used. Before cryopreservation, semen samples from each ejaculate were divided into 4 aliquots and extended with Tris alone or supplemented with 1 of 3 different concentrations of IGF-I (50, 100 and 250 ng/mL). Semen was evaluated immediately after thawing (T0), after 1 h (T1) and 2 h post-incubation at 37°C. The percentage of morphologically normal spermatozoa and live cells (fluorescence analysis-calcein and ethidium), acrosome integrity (NAR) and motility were analysed and hypoosmotic swelling test (HOST) was performed to evaluate in vitro sperm survival. This experiment was replicated 5 times. In addition, AI was performed using 121 ewes to compare the optimal concentration of IGF-I versus Tris alone on fertility. Data for sperm parameters were analysed by 1-way ANOVA and the differences among groups were examined by Duncan’s multiple range test. Pregnancy rates were analysed by the chi-square test of independence.

After 1 and 2 h post-incubation, a decrease was observed in all groups in the percentage of motility, NAR and HOST when compared to the semen at T0. The motility rate was higher (P < 0.05) in the IGF-I 100 and IGF-I 250 groups when compared to the IGF-I 50 and Tris alone groups (76.2 and 75% vs 66.2 and 64.4%, respectively) at T0, after 1 h (67 and 63.6% vs 57.4 and 56.2%) and 2 h post-incubation (58.2 and 55.4% vs 48 and 47.2%). Furthermore, viability was higher (P < 0.05) in the IGF-I 100 and IGF-I 250 groups than in the IGF-I 50 and Tris alone groups (88.7 and 88.3% vs 76.6 and 77.6%, respectively) at T0. There was no difference (P > 0.05) in NAR or HOST among groups. No significant differences (P > 0.05) in fertility were observed between IGF-I 100 and Tris groups. In conclusion, the supplementation of Tris extender with IGF-I (100 and 250 ng/mL) appears to be a successful way to preserve functional parameters of ovine semen during cryopreservation.

48 EFFECT OF TREHALOSE ADDITION ON IN VITRO VIABILITY OF COOLED RAM SPERMATOZOA

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Cryopreservation of ovine spermatozoa is a valuable tool for genetic progress. Sugars, in particular disaccharides, have been shown to have positive effects in semen extender, providing energy substrate during cell incubation, decreasing osmotic pressure, causing cell dehydration and ultimately reducing cold shock and cryodamage. Several authors have reported changes in plasma membrane protein organisation and membrane fluidity during cooling. Trehalose, a non-reducing disaccharide has been associated with stabilisation of cellular proteins and interaction with membrane phospholipids due to its ability to replace water at the membrane-solution interface. Many studies have confirmed the beneficial effect of the addition of trehalose during cryopreservation at –196°C; however, its effects during refrigeration (5–7°C) have not been reported. Ejaculates form four fertile Corriedale rams were collected by electroejaculation during the ovine reproductive season in the Southern Hemisphere (May–August); the study was conducted in 12 replicates. After initial quality assessment, ejaculates were pooled into heterospermic samples, diluted in Tris-buffered extender and separated into treatments consisting of (a) no refrigeration (kept at room temperature 22–25°C), (b) refrigeration (cooling rate of 2°C/3 min to reach a final temperature of 5–7°C), or (c) refrigeration with the addition of 150 mM trehalose. Samples were evaluated for total motility, progressive motility, hypoosmotic swelling test (HOST) and eosin Y (1 step, WHO protocol); live/dead stain at 0, 6, 12, 24 and 48 h post-refrigeration. Results were analysed using 1-way ANOVA with Tukey post-hoc multiple comparison test. Compared with refrigeration without the addition of disaccharides, the refrigeration in presence of trehalose significantly increased (P < 0.05) total motility at 48 h (32 ± 0.9 vs 50 ± 1.1) and progressive motility at 24 h (40 ± 0.9 vs 65 ± 0.6) and 48 h (0 vs 38 ± 1.0) post-refrigeration. Trehalose also decreased (P < 0.05) the percentage of damaged spermatozoa as evidenced by HOST and live/dead stain after 12 h (42 ± 1.1 vs 29 ± 0.7), 24 h (52 ± 0.9 vs 37 ± 0.6) and 48 h (74 ± 0.8 vs 60 ± 1.0) of refrigeration. Although no statistical difference was detected, there was a trend of trehalose to improve total and progressive motility parameters at the initiation of treatments (0 h). In addition, spermatozoa refrigerated in the presence of trehalose was the only treatment group to retain progressive motility after 48 h of storage. Our results indicate that trehalose could be used to extend the in vitro viability of refrigerated ovine spermatozoa for up to 48 h. Further studies are needed to assess the in vivo fertility (pregnancy rate) of refrigerated spermatozoa in the presence of this non-permeating cryoprotectant sugar.

49 USE OF DILUTION OF CRYOPRESERVED SEMEN IN FIXED-TIME ARTIFICIAL INSEMINATION OF MANGALARGA MARCHADOR MARES


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Equine AI with frozen semen has become more frequent in reproduction centers, but osmotic shock during thawing is still a major problem as it impairs gamete viability and fertility. To overcome this, ultrasonicographic (US) exams must be performed to ensure AI at the closest time to...
Ovulation possible. The objective was to evaluate, in vivo, the effects of dilution of the cryoprotectant dimethylphormamide on the success of a fixed timed (20 h post-ovulation) AI protocol, in an attempt to reduce osmotic shock and the intense labour usually required during equine AI with frozen semen. Thirty-five Mangalarga Marchador mares were used in this trial. When dominant follicles reached at least 35 mm in diameter, ovulation was induced with 6 mg of equine gonadotrophin extract (EGE) intravenously. Artificial insemination was performed 20 h after ovulation according to 1 of the following protocols: no dilution (n = 14): three 0.5-mL straws were thawed and the semen deposited by a flexible rod at the uterine tip in the ipsilateral horn where the pre-ovulatory follicle was detected; or dilution (n = 21): three 0.5-mL straws were thawed as described and centrifuged after addition of 1.5 mL of BotuSiemen® extender (1:1 dilution). Pregnancy rates were evaluated between treatments, age and presence of intrauterine liquid (IUL) classes and period between ovulation and AI. Data were analysed as a binomial distribution with the GENMOD procedure (SAS, Cary, NC, USA). Pregnancy rate was higher (P < 0.05) for mares inseminated with diluted semen (42.9%) compared to those inseminated with no semen dilution (48.8%). Age and IUL class did not influence pregnancy rates. It can be concluded that reduction of osmotic stress through the dilution of cryopreserved equine semen increased pregnancy rates after AI. Semen dilution, as tested in this trial, yielded good pregnancy rates in a fixed-time AI protocol in mares, simplified mare management greatly and allowed the use of a fixed-time protocol successfully.

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50 EFFECTS OF VITAMINS ON THE QUALITY AND FERTILITY OF BOAR SEMEN AFTER LIQUID PRESERVATION AT 5°C

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Liquid preservation can be used as an alternative to freeze-thawing for preserving semen for AI. The efficiency of some boar semen extenders has been studied over storage periods of 5 to 7 days. The objective of this study was to evaluate the viability and penetrability of boar spermatozoa preserved at 5°C in a modified Modena-based extender supplemented with either 100 μM vitamin C (Vc), 100 μM vitamin E (Ve), or 100 μM Ve + 100 μM Ve (Ve + e). The final sperm concentration was adjusted to cells mL⁻¹ and the semen was then stored at 5°C for 4 weeks. In Experiment 1, the semen samples were assessed every week during the 4-week storage in each extender for the following factors: motility, by using computer-assisted semen analysis (CASA); viability, by using the Live/Dead fluorescence viability assay; plasma membrane integrity, by using the hypotonic swelling test (HOST); and acrosome integrity, by using fluorescein isothiocyanate (FITC)-labelled peanut agglutinin staining. In Experiment 2, we examined the penetrability of spermatozoa that had been stored in each extender for 4 weeks and the development of fertilized oocytes. Data were analysed using ANOVA. In Experiment 1, when the semen was stored for 2 weeks, the mean percentage values of total sperm motility and viability for semen stored with Ve were significantly higher than those for semen stored without Vc and Ve (control group) (84.3 vs 67.9% and 59.8 vs 51.2%, respectively; P < 0.05). Moreover, the percentage sperm motility for semen stored for 4 weeks tended to be higher in the Ve group than in the control group (44.2 vs 32.7%; P < 0.1). Storage with Vc or Ve + e did not improve sperm motility and viability of semen. The plasma membrane integrity and acrosome integrity of semen did not significantly differ among the groups during the 4-week storage. In Experiment 2, the rates of sperm penetration and of development to blastocysts of fertilized oocytes did not differ between the Ve and control groups (33.0 vs 28.5% and 14.9 vs 10.1%, respectively; P > 0.05). However, storage with Vc reduced the rate of oocyte development compared with the Ve and control groups (1.1%; P < 0.05). In conclusion, adding Ve to the semen extender may improve the motility and fertility of boar semen stored at 5°C. However, adding Vc has a harmful effect on the quality and fertility of stored boar semen.

51 CHANGING ROOSTER SPERM MEMBRANES TO FACILITATE CRYOPRESERVATION

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Cryopreservation damages rooster sperm membranes. Part of this damage is due to membrane transitioning from the fluid to the gel state as temperature is reduced. Some of this damage may be prevented by increasing membrane fluidity at low temperatures by incorporating cholesterol or unsaturated lipids into the membrane. Different concentrations of cholesterol-loaded cyclodextrins (CLC) and lipid-loaded cyclodextrins (LLC) containing 1,2-dilinoleoyl-sn-glycero-3-phosphocholine; 1,2-dilinoleoyl-rac-glyceryl; and 1,2-dilinolenoyl-sn-glycero-3-phosphocholine were added to rooster sperm to determine if they improved cryopreservation. Osmotic stresses when cryoprotectants (CPA) are added to the cells before freezing and when the CPA are removed from cells after thawing also cause membrane damage. To minimize this damage, low molecular weight CPA with high membrane permeability were tested to determine their effectiveness for cryopreserving sperm. Rooster semen was collected from several birds, pooled and diluted to 800 million sperm mL⁻¹ at 5°C in Lake’s Low Temperature diluent (LLT). Sperm were treated with either LLC (0.25, 0.5, 1, 1.5, 2, 4 and 6 mg mL⁻¹) or CLC (0.5, 1 and 2 mg mL⁻¹) for 30 min. The sperm were diluted 1:1 with LLT containing 18% CPA, resulting in final CPA concentrations of 9%. The CPA tested were glycerol (G), methylacetamide (MA), dimethylformamide (DMF), methylformamide (MF) and ethylene glycol (EG). The sperm were frozen in liquid nitrogen vapor and stored in liquid nitrogen. Straws were thawed in 5°C water and sperm motility and membrane integrity analysed immediately. Sperm motility was measured using computer-assisted sperm analysis (CASA) and membrane integrity was analysed by flow cytometry using propidium iodide to detect cells with damaged membranes. Data were analysed by ANOVA and means separated using Student–Newman–Keuls multiple comparison test. Addition of LLC and CLC did improve sperm cryosurvival rates (P > 0.05). Using G as the CPA resulted in higher percentages of motile (54%) and viable (58%) sperm than MA (47 and 52%; P < 0.05), whereas DMF, EG and MF resulted in less than 45% motile cells (P < 0.05). In conclusion, altering sperm membrane composition
using CLC and LLC did not improve post-thaw motility or viability in rooster sperm. Although MA did not protect the rooster sperm from cryodamage as effectively as G, future assays will need to determine the fertilizing capacity of sperm frozen using these CPA.

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52 HYALURONIC ACID IMPROVES CRYOTOLERANCE OF BUFFALO (BUBALUS BUBALIS) IN VITRO-DERIVED EMBRYOS


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Although in vitro embryo production efficiency in buffalos has greatly improved over the years, the in vitro-produced embryos show lower viability and resistance to cryopreservation. Therefore, it is necessary to optimize the in vitro culture conditions to improve embryo quality. Hyaluronic acid, a glycosaminoglycan present in oviducal and uterine fluids, has been shown to successfully support in vitro development of bovine embryos (Stojkovic et al. 2002 Reproduction 124, 141–153). The aim of this study was to evaluate the influence of high concentrations of hyaluronic acid (HA) during late in vitro culture on blastocyst development, as well as on their cryotolerance after cryoprotectant vitrification in buffalos. In vitro matured and fertilized buffalo oocytes (n = 1007) from slaughterhouse ovaries were cultured for 4 days in SOFaa supplemented by 8 mg mL⁻¹ of BSA in a controlled gas atmosphere consisting of 5% CO₂, 7% O₂ and 88% N₂, in humidified air, at 38.5°C. On Day 4, cleavage rate was assessed (75.2%) and all of the cleaved elements were divided into 3 different late culture groups: 8 mg mL⁻¹ of BSA (n = 244; group A), 8 mg mL⁻¹ of BSA supplemented by 6 mg mL⁻¹ of HA (n = 251; group B) and 1 mg mL⁻¹ of BSA supplemented by 6 mg mL⁻¹ of HA (n = 262; group C). On Day 7 after IVF, embryo outcome was assessed and all of the embryos were vitrified by cryoprotectant [De Rosa et al. 2007 Ital. J. Anim. Sci. 6 (Suppl 2), 747–750] and cultured for 24 h. The resistance to cryopreservation was evaluated by assessing the survival rate on the basis of morphological criteria and the percentage of embryos reaching a more advanced developmental stage after 24 h culture. Data were analysed by the chi-square test. No differences in blastocyst rate were recorded among groups (43.9, 44.3 and 40.0%, respectively in A, B and C groups). However, out of the total embryos, a higher percentage of Grade 1 hatched blastocysts (Robertson and Nelson 1998 Manual of the International Embryo Transfer Society 9, 103–16) was observed in group C (P < 0.05) than in groups A and B (14.3, 18.8 and 25.5% in A, B and C groups, respectively). Although the supplementation with HA did not improve the survival rates following vitrification-warming (51.1, 59.4 and 58.4% in A, B and C groups, respectively), the percentage of vitrified-warmed embryos that resumed development and reached a more advanced developmental stage after culture increased (P < 0.01) in group C (20.7, 27.7 and 37.6% in A, B and C groups, respectively). In conclusion, the addition of 6 mg mL⁻¹ of HA, together with a limited protein source (i.e. 1 mg mL⁻¹ of BSA), during late culture improved buffalo embryo quality, indicated by both the greater percentage of advanced-stage embryos and by the resumption of development after post-warming culture.

53 VITRIFICATION OF HANDMADE CLONED BUFFALO EMBRYOS USING ETHYLENE GLYCOL AND DIMETHYL SULPHOXIDE AND SUBSEQUENT EFFECT ON CRYOSURVIVAL AND APOPTOSIS


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The objective of the present work was to optimize concentration of cryoprotectants ethylene glycol (EG) and dimethyl sulphoxide (DMSO) to facilitate the vitrification of buffalo embryos produced by somatic cell nuclear transfer (SCNT). Cloned embryos were produced according to standardized protocols of our laboratory using handmade cloning (Shah et al. 2008). Three different concentrations of EG and DMSO (7.5, 10 and 15%, having 0.5 M sucrose in TCM-199 containing 20% serum) were selected in combination for vitrification of cloned blastocysts in French ministraws (0.25 mL). The numbers of cloned blastocysts vitrified for each concentration were 51, 51 and 52, respectively. The post-thaw viability was accessed by re-expansion rate of blastocysts after culturing in RVCL media (K-RVCL-50, Cook® Australia, Queensland, Australia) for 18 to 24 h. On the basis of re-expansion rate, there was no significant effect of any selected concentrations (7.5, 10 and 15%) on post-thaw viability (25.33 ± 2.43%, 29.00 ± 2.52% and 30.83 ± 3.01%, respectively; P > 0.05). The effect of vitrification on apoptosis level was checked after 18 to 24 h post-thaw by TUNEL assay and the apoptosis index was calculated by dividing the total number of nuclei with DNA-fragmented positive nuclei of the respective blastocyst. We found that, 7.5% group embryos resulted with a significantly higher apoptotic index (8.28 ± 0.57) than that of the 10 and 15% groups (5.09 ± 0.46 and 4.28 ± 0.24, respectively; P < 0.05). These results clearly indicate that a lower concentration of cryoprotectants (7.5%) increased the chance of apoptosis in blastocysts that were frozen-thawed. The quantitative expression of apoptosis-related genes (Bax, Bid, Mc-I-1 and Bcl-xI) in all 3 treatment groups and fresh control embryos were determined by RT-qPCR. Three replications were performed and the mRNA level of each sample was normalized to that of glyceraldehyde-3-phosphate dehydrogenase mRNA level. Results of RT-qPCR were analysed using the 2⁻ΔACT method to compare the relative transcriptional levels of the target genes in each group. The RT-qPCR data revealed that the 7.5% vitrified group embryos possessed high expression of pro-apoptotic genes (Bax and Bid) and lower expression of anti-apoptotic genes (Mc-I-1 and Bcl-xI) in comparison to the 10 and 15% groups. However, there was no significant change in gene expression between the 10 or 15% groups in comparison with fresh non-vitrified embryos. Our results conclude that the best choice is to use 10 or 15% EG and DMSO cryoprotectants for in-straw zona-free cloned buffalo embryo vitrification. However, further experiments are needed to enhance survival after vitrification.

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54 CRYOPRESERVATION OF DOMESTIC CAT EPIDIDYMAL SPERM IN A DEFINED EXTENDER WITHOUT ANIMAL OR PLANT PROTEINS

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Previously, we have shown that survival of cat sperm is maintained in both non-egg yolk, semi-defined extenders and in extenders with greatly reduced levels of egg yolk (2%). Usually, cryoprotectant is added to extended samples after gradual cooling to 4°C, but recent reports have shown that satisfactory sperm survival can be obtained after addition at 22°C. Here, our objectives were to examine sperm survival after (1) cryopreservation from 22°C vs after gradually cooling to 4°C or (2) cryopreservation in a completely defined extender without animal or plant proteins vs extender + 2% egg yolk. Epididymides from local veterinary clinics were dissected in HEPESS 199 medium (He199). The sperm suspension was filtered (40 μm), layered onto a density gradient column and centrifuged at 650 × g for 20 min. Then, the sperm pellet was resuspended in 1 mL of He199 and centrifuged for 5 min at 800 × g and the subsequent pellet was extended in TEST Buffer with either 0% (0% EY) or 2% egg yolk (2% EY). Next, 0% EY samples were further split into 2 groups—either gradually cooled to 4°C before 12% glycerol (1:1) was added (4C-0%EY) or 12% glycerol (1:1) was added at 22°C without cooling (22C-0%EY). Control samples extended in 2% EY were cooled to 4°C before addition of 12% glycerol (1:1)(4C-2%EY). Samples were loaded into 0.25-ml straws and placed in a ~80°C freezer for 20 min before storage in LN2. Sperm samples were thawed in air (22°C) for 5 s and immersed in a 60°C water bath for 5 s. After a 7-step addition of He199, samples were centrifuged at 800 × g for 5 min and pellets resuspended in He199. Sperm samples were evaluated for motility (Mot; computer-assisted semen analysis, 37°C) at 0 h (initial assessment), after cooling to 4°C (PC) and at 0-PT (0-PT) and 3-PT (3-PT) post-thaw (3-PT) incubation at 37°C. Membrane integrity (MI; SYBR 14-PI) and acrosomal status (AS; FITC-PNA) were analysed at the initial assessment, 0-PT and 3-PT. Results are shown in Table 1. At 4°C (PC), sperm extended in 0% EY and 2% EY maintained 92 and 91%, respectively, of their initial motility (66%). At 0-PT and 3-PT, motility in the 3 groups had decreased by >50% and >70%, respectively. Motility at 3-PT in the 22C-0%EY treatment was less than the other 2 treatments (P < 0.05; 1-way ANOVA). At 0-PT, sperm in the 4C-2%EY group had a higher membrane integrity value (P < 0.05) than did the 22C-0%EY group, whereas that of the 4C-0%EY group was not different from the other 2 groups. However, at 3-PT, both groups cooled to 4°C before cryopreservation had higher membrane integrity values (P < 0.05) than the group cryopreserved at 22°C. At 0-PT and 3-PT, the percentage of sperm with intact acrosomes ranged from 69% (4C-2%EY) to 59% (22C-0%EY) and from 55% (4C-2%EY) to 43% (22C-0%EY) of the initial value (98%), respectively. In summary, we demonstrated that cat epididymal sperm could be frozen successfully in a completely defined TEST-buffered extender. Furthermore, we confirmed that addition of cryoprotectant (i.e. glycerol) after gradual cooling to 4°C is beneficial to post-thaw survival.

Table 1. Motility (Mot), membrane integrity (MI) and acrosomal status (AS) of cat epididymal sperm before and after cryostorage

<table>
<thead>
<tr>
<th>Treatment</th>
<th>PC</th>
<th>Mot, % (66.0%)*</th>
<th>MI, % (80.0%)*</th>
<th>AS, % (89.0%)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PT 0 h</td>
<td>PT 3 h</td>
<td>PT 0 h</td>
<td>PT 3 h</td>
</tr>
<tr>
<td>4C-2%EY</td>
<td>61.0</td>
<td>31.0</td>
<td>18.0\textsuperscript{a}</td>
<td>52.0\textsuperscript{a}</td>
</tr>
<tr>
<td>4C-0%EY</td>
<td>60.0</td>
<td>26.0</td>
<td>15.0\textsuperscript{a}</td>
<td>50.0\textsuperscript{b}</td>
</tr>
<tr>
<td>22C-0%EY</td>
<td>23.0</td>
<td>9.0\textsuperscript{b}</td>
<td>41.0\textsuperscript{b}</td>
<td>33.0\textsuperscript{b}</td>
</tr>
</tbody>
</table>

\textsuperscript{a,b}Means with different superscripts are significantly different (P < 0.05); \*initial value; PC = post-cool; PT = post-thaw.

55 PROPAGATION OF MULTIPLE CAT HEREDITARY DISEASE MODELS FOLLOWING ASSISTED REPRODUCTION WITH FROZEN SEMEN AND EMBRYOS

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Domestic cats are invaluable research models for the study of hereditary diseases that affect both cats and humans. By necessity, most cat models are maintained as living populations; however, semen and embryo cryopreservation could provide a cost-effective alternative for model conservation if viable offspring can be readily produced with assisted reproductive techniques such as IVF, embryo transfer (ET) and AI. In our earlier research, semen and IVF-derived embryos representing 24 cat models at 7 veterinary/medical schools were frozen for liquid nitrogen storage. Our objectives in this study were to (1) assess the application of assisted reproduction using frozen semen or embryos for producing pregnancies and viable kittens in several cat models and (2) provide our university collaborators with rederived model offspring. Five cat models (i.e. spinal muscular atrophy, SMA; porphyria, POR; Chediak-Higashi syndrome, CHS; progressive retinal atrophy, PRA; and hypertrophic cardiomyopathy, HCM) were selected for propagation based on investigator research needs. For 3 models (SMA, POR and CHS), semen from affected males, frozen as pellets in Test Egg Yolk medium (4% glycerol), was thawed and used to inseminate (5 × 10^5) motile sperm mL\textsuperscript{-1} oocytes from eCG/hCG-treated queens. Resulting embryos (2- to 8-cell stage) were transferred laparoscopically (3-5 embryos/recipient) into the oviducts of anestrous queens synchronized with eCG/pLH. For PRA, IVF
The oviduct provides the environment necessary for the gamete transport, completion of spermatozoa capacitation, oocyte fertilization and the early development of embryos. In cattle, all of these processes take place between Day 0 to 4 of the ovarian cycle (Day 0 is the day of ovulation). In previous studies, temporal changes in the bovine oviduct morphology were evaluated by dividing the ovarian cycle into luteal and follicular phases. In order to understand the relation between the bovine oviduct morphology and processes occurring there, the ovarian cycle has been further divided into four phases: I (Day 0–4), II (Day 5–10), III (Day 11–17) and IV (Day 18–20), with the day of ovulation considered Day 0 (1980 J. Dairy Sci. 63, 155–160). The aim of the study was to evaluate the oviduct morphology of the infundibulum, ampulla and isthmus in 4 phases of the ovarian cycle. Research material comprised cattle oviducts (classified into 1 of the 4 phases of the cycle based on ovarian morphology), dissected into infundibulum, ampulla and isthmus and subsequently sectioned and processed for histological preparations (hematoxylin and eosin, H&E, staining). Diameters of transverse cross-sections of oviduct and its lumen and thickness of tunics: mucosa, muscularis and serosa were evaluated in relation to the region of oviduct and the phase of ovarian cycle. Values are given in μm. Statistical analysis was carried out by 1-way analysis of variance and comparisons of mean values were made with the Tukey honestly significant difference test (Statgraphics Plus 5); P < 0.05 was considered to reflect the presence of statistical significance. The comparison of the diameters of transverse cross-sections (A) of oviduct and its lumen (B) shows significant statistical differences between ampulla and isthmus within the phases: A-I (4507.26 vs 2524.47), II (4510.53 vs 2540.67), III (4503.28 vs 2534.07), IV (4500.73 vs 2533.90); B-I (4191.10 vs 1950.88), II (4173.63 vs 1986.33), III (4198.53 vs 1966.88) and IV (4192.50 vs 1939.33). There are no differences among 4 phases of the ovarian cycle. The thickness of tunica muscularis and serosa of infundibulum (I: 26.81 vs 196.85; II: 27.03 vs 201.80; III: 26.22 vs 199.45; IV: 23.97 vs 198.01), ampulla (I: 91.51 vs 214.50; II: 90.72 vs 212.55; III: 88.61 vs 213.30; IV: 89.65 vs 206.28) and isthmus (I: 202.29 vs 216.52; II: 199.24 vs 207.74; III: 200.90 vs 212.38; IV: 200.38 vs 210.86) show only statistically significant differences within the phases, whereas the tunica mucosa shows only statistically significant differences between phases and the term of the height of epithelium at the base of mucosal folds (I: 26.49; II: 25.20; III: 24.14; IV: 29.96) and their apical parts (I: 28.09; II: 26.01; III: 25.45; IV: 30.96). In conclusion, differences in oviduct morphology are mainly region specific, whereas the epithelium morphologically infundibulum, ampulla and isthmus show variation in the 4 phases of the ovarian cycle.

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56 COMPARISON OF THE MORPHOLOGY OF VARIOUS REGIONS OF THE CATTLE OVIDUCT IN FOUR PHASES OF THE OVARIAN CYCLE


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Shortly after parturition the metabolic situation of high-yielding dairy cows is often dominated by a negative energy balance. These effects affect the whole animal and may especially be detected in the reproductive tract, where they result in reduced fertility. An oral supplementation with dietary fats is often used to counteract by reducing milk fat content and, thus, supplying the individual animal with an increased amount of energy. The focus of the present study was to analyse the effects of an oral supplementation with conjugated linoleic acids (CLA) on corpus luteum (CL) function. Healthy Holstein-Friesian cows and heifers were randomly allocated to 2 treatment groups (Group 1: 50 g of CLA day⁻¹ per animal, 2 heifers, 6 cows;