

after transfection. Then we tried to suppress endogenous gene Oct4 that plays an important role in the maintenance of pluripotency and the lineage commitment in mouse ES cells. We performed RT-PCR analysis and western blotting to assay for Oct4 mRNA and protein at 24, 48, 72, and 96 h after transfection. Hand1 and Cdx2, transcription factors implicated in trophoblast differentiation, were also analyzed. RT-PCR analysis showed a reduced level of Oct4 mRNA at 24–96 h. Reduction of Oct4 protein was confirmed by western blotting and the reduced level was still maintained at 96 h after transfection. RT-PCR analysis also showed up-regulation of Hand1 and Cdx2 concomitant with the suppression of Oct4. Furthermore, ES cells changed their morphology into a large and flattened shape that is characteristic of trophoblast cells. These results suggested that the transient suppression of Oct4 induced differentiation of mouse ES cells to trophoblast cells as expected. Therefore our data imply that the chemically synthesized siRNA can be used to differentiate ES cells. Next, we tried to suppress EGFP and Oct4 in monkey ES cells. In monkey ES cells, we found that the Sendai virus (hemagglutinating virus of Japan, HVJ) envelope was suitable to deliver siRNA into cells. With this method, we detected efficient silencing of EGFP and Oct4 by fluorescence microscopy, RT-PCR analysis, and western blotting. In the case of monkey ES cells, however, no morphological change was observed by Oct4 suppression at 96 h after transfection. These results suggest possible diversity between murine and primate ES cells in the differentiation process.

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## 181 Oct-4: A POTENTIAL MARKER FOR PLURIPOTENCY IN CATTLE

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The POU (Pit-Oct-Unc) domain transcription factor Oct-4 is one of the most acknowledged markers for pluripotency in murine and primate embryonic cells. At the blastocyst stage, expression of Oct-4 has been shown to remain high in the inner cell mass (ICM) while being rapidly down regulated in the trophoblast (TE). Furthermore, in these species, expression of Oct-4 is maintained in pluripotent derivatives of the ICM and in embryonic stem (ES) cells, but lost upon differentiation. In the bovine embryo, a marker with similar qualities has long been sought. The aim of this study was to investigate, using a commercially available antibody for immunohistochemistry (IHC), whether Oct-4 might serve this role. *In vitro* produced (IVP) embryos were transferred to synchronized recipients at Day 6 post insemination (p.i) and flushed at Day 12; *in vivo*-derived embryos were flushed at Day 14. Day 8 IVP embryos ( $n = 20$ ) were fixed and processed for IHC in paraffin sections together with Day 12 ( $n = 18$ ) and Day 14 ( $n = 3$ ) embryos. From Day 8 IVP embryos, outgrowth colonies (OCs) were formed by intact blastocyst culture on mouse SNL feeder cells. OCs were photographed using a stereomicroscope on Days 12, 14, and 16 p.i., and were examined for Oct-4 expression by *in situ* IHC at Day 16 p.i. ( $n = 94$ ). From isolated embryonic discs of Day 12 embryos, OCs were derived by similar culture and were either processed for IHC on paraffin sections at Days 16, 18, and 20 p.i. ( $n = 9$ ) or used for establishment of ES-like cell lines. Of colonies formed, representative specimens from each of the initial 5 passages ( $n = 18$ ) were examined for Oct-4 expression either in paraffin sections or *in situ*. In Day 8 embryos, Oct-4 expression was demonstrated in all nuclei of both ICM and TE cells except for presumptive apoptotic ones. Approximately one-fifth of the OCs presented a substantial amount of Oct-4 positive cells of putative ICM, but also of TE origin. Apparently, the formation of Oct-4 positive OCs was favored by initial attachment of the embryonic pole to the feeder cells. In Day 12 and 14 embryos, specific and exclusive Oct-4 staining of nuclei of the complete epiblast, but not the hypoblast and the TE, was revealed. All OCs derived from Day 12 embryonic discs showed specific staining for Oct-4 in nuclei of putative epiblast origin only. On subsequent culture of these isolated epiblast derivatives, loss of Oct-4 staining from colonies was observed by passage 3. This study has, for the first time, shown expression of Oct-4 to be limited to pluripotent cells of bovine Day 12 and 14 embryos. Compared with murine and primate embryos, down-regulation of Oct-4 expression in bovine TE cells appears to be delayed. Findings indicate that Oct-4 may be used as a marker for pluripotency in bovine ES-like cells, although TE derivatives may maintain Oct-4 expression when isolated from Day 8 embryos.

*Epidemiology/Diseases*

## 182 DISINFECTION OF DRY (VAPOR) SHIPPERS (“DEWARS”) FROM MICROBIAL CONTAMINATION ASSOCIATED WITH CRYOPRESERVED GERMPLASM

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Cryopreservation, storage, and transport of cryopreserved germplasm without the risk of disease transmission is of great concern to animal and human health authorities. Here we report on the efficacy of microbial decontamination of liquid nitrogen (LN) dry (vapor) shippers used for short-term storage and transportation of germplasm and other biological specimens. Dry shippers containing either a hydrophobic or a nonhydrophobic LN absorbent were experimentally contaminated with high titers of cultures of *Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus aureus*, bovine viral diarrhea virus (BVDV), and bovine herpesvirus-1 (BHV-1). Biocides with broad-spectrum antimicrobial activity and gas vapors of formalin and ethylene oxide were used for disinfection of the shippers. All biocide solutions were diluted with milli-Q water to the concentrations recommended by the manufacturer and poured directly into the chamber of the dry shippers. The dry shipper was filled with the disinfectant for 30 min, drained, washed three times with sterile water, and drained before testing the residue for microbial contaminant. Among the biocides used, treatment with sodium hypochlorite solution (30% of household bleach), a quaternary ammonium-based disinfectant (100% Expel), and peracetic acid (30%) were the most effective and useful for dry shippers with a hydrophobic LN absorbent. None of the bacterial or viral microorganisms were detected in samples of semen and embryos stored in dry shippers following their disinfection with these biocides. Other disinfectants (Virkon, Roccal,

1-Stroke, Expel Odor) were not effective due to their foaming properties. Some other disinfectants (Viralex and 70% ethanol) caused irreversible damage to the permeability of the LN-absorbent hydrophobic membrane. Gas sterilization by ethylene oxide was effective for both types of dry shipper.

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### 183 EFFECT OF THE ACIDIC ORGANIC BUFFER MES ON BOVINE *IN VITRO* EMBRYO PRODUCTION

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Transferred embryos carry the risk of being vehicles of organisms causing diseases. Currently, the risk of *in vitro*-produced (IVP) embryos is more difficult to assess than the risk of *in vivo*-derived embryos, since less research has been published on the former. Foot and mouth disease virus (FMDV) is extremely sensitive to a low pH and is likely to be destroyed if embryos are exposed to a low pH for a short time. 2-(N-Morpholino)-ethanesulfonic acid (MES); an organic buffer with pKa 6.1; Sigma, South Africa, M2933) as been shown to destroy FMDV at a rate of 90% per minute at pH 6 and at a rate of 90% per second at pH 5 (Acharya *et al.* 1990 Vet. Microbiol. 23, 21–34; Thomson “Foot-and-mouth disease,” in *Infectious Diseases of Livestock with Special Reference to Southern Africa*, ed. Coetzer JAW, Thomson GR, and Tustin RC, Oxford University Press, Cape Town, 825–852). The aim of this study was to test whether exposing bovine oocytes and IVP zygotes to the organic buffer MES, buffered at pH 5.5, is detrimental to the development of bovine IVP embryos. IVM, IVF, and IVC was carried out with 1367 oocytes as described earlier [Jooste *et al.* 2003 Theriogenology 59, 443]. Oocytes were divided into three groups: 484 were used as controls (no MES exposure); 437 were in a maximal exposure group (MAX), i.e. MES treatment after washing of oocytes, after IVM and after IVF, and 446 had a minimal exposure (MIN), i.e. MES treatment after IVF only. To treat the oocytes with MES, 100 oocytes (from ten droplets) were drawn into a pipette in a maximal volume of 100  $\mu$ L, and placed in 3 mL of MES, swirled around for 10 s, drawn up again in a maximal volume of 100  $\mu$ L, and placed in 3 mL of culture medium. Oocytes or zygotes were then washed five times in culture medium before being processed through IVM, IVF, or IVC depending on their stage. Exposure of oocytes to MES varied from 30 to 60 s (10 s swirling and a variable time thereafter to pick up). A chi-square test was used to test for differences in cleavage and Day 7 blastocyst yield between control and treatment groups ( $P < 0.05$ ). Cleavage (70%; 340/484) and blastocyst yield (32%; 156/484) in the control group were not different from those in MIN (68%; 304/446, and 29%; 131/446, respectively), but were significantly higher than for MAX (57%; 249/437, and 18%; 79/437, respectively). In MAX the MES had a harsh effect on the cumulus cells, making them granular and clumpy in appearance. Oocytes treated in MES solution adhered to the bottom of the dish, which made their handling difficult. Exposure time in MES was therefore variable and longer than initially planned. It is concluded that bovine IVP embryos can be exposed to MES without detrimental effect. Treatment with MAX still resulted in blastocysts but it did not yield good numbers. In future trials, treated dishes should be used to prevent oocyte and zygote adherence. Further research is needed to test whether FMDV can be removed from bovine IVP embryos with the described method.

### 184 THE EFFECT OF A NOVEL SEMEN DISINFECTION TREATMENT ON THE VIABILITY AND FERTILIZING CAPACITY *IN VIVO* OF BOVINE SPERMATOZOA

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The goal of this investigation was to determine the effect of a novel procedure, consisting of an initial incubation in antibiotic cocktail followed by centrifugation through a trypsinized Percoll gradient, on the viability and fertilizing capacity *in vivo* of bovine sperm as compared to standard processing methods. Exp 1: Semen collected by electroejaculation from 12 bulls were aliquoted into four treatment groups: (1) control (standard method) using Biladyl A (Minitübe, Tiefenbach, Germany) at a 1:4 (semen:diluent) dilution; (2) antibiotic cocktail (gentamicin, spectinomycin, lincomycin, tylosin, and kanamycin at 250, 300, 150, 200, and 1000  $\mu$ g/mL, respectively) at a 1:9 (semen:cocktail) dilution and incubation (38°C) for 15 min; (3) trypsinized Percoll (Sigma, St. Louis MO, USA) gradient treatment by layering 1 mL semen on the top of a 90, 45, and 30% Percoll (bottom to top) gradient, with the 90 and 30% segments of the column containing 10  $\mu$ g/mL soy-based trypsin inactivator (Sigma) and the 45% segment containing 0.25% trypsin (Sigma), and then centrifuging at 700g for 30 min; and (4) a combination of treatments 2 and 3 by concentrating the sperm by gentle centrifugation (300g for 10 min) after incubation in antibiotic cocktail, and then layering 1 mL of the sperm suspension on the Percoll gradients. In treatments 2–4, the treated sperm pellets were resuspended in Biladyl A (1:4), and all treatments were refrigerated (4°C) and examined at 0, 24, 48, and 72 hours for progressive motility, viability, and acrosomal integrity. The results were analyzed statistically using the Student's *t*-test ( $P < 0.05$ ). Exp 2: Semen samples collected from two bulls were treated with either the control method or the combination antibiotic cocktail and trypsinized Percoll methods (1 and 4 above), and then used to AI a total of 5 (control) and 6 (combined treatment) superovulated cows three times at 12-h intervals. Day 7 embryos were recovered and assessed for stage and morphological quality. The results for Exp 1 indicated that sperm treated with either the trypsinized Percoll alone or in combination with the antibiotic cocktail had significantly higher ( $P < 0.05$ ) progressive motility than the control at 0 (87.5 and 89.2 vs. 75.4%), 24 (87.1 and 87.9 vs. 73.2%), 48 (87.1 and 86.7 vs. 70.7%), and 72 h (82.1 and 80.4 vs. 64.6%, all respectively) post-treatment. Likewise, treated sperm had significantly greater viable and acrosome-intact sperm than the control at 0 to 72 h post-treatment. Although not statistically significant, there were more transferable-quality embryos recovered from cows inseminated using sperm treated with the combined method as compared to the control (58.9 vs. 43%, respectively). In conclusion, the trypsinized Percoll gradient method for processing bovine semen (alone or in combination with antibiotic cocktail) improved the quality of bovine sperm refrigerated for up to 72 h and had no detrimental affect on the number of transferable-quality embryos collected after AI.

# 185 A NOVEL AND EFFECTIVE PROCEDURE FOR REMOVING HIV-1 AND HEPATITIS B AND C VIRUSES FROM SPIKED HUMAN SEMEN

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The objectives of this study were to determine the effectiveness of a novel, trypsinized density gradient treatment designed to remove viruses from semen and to evaluate sperm viability after treatment. Exp. 1: Cryopreserved human semen ( $n = 6$  donors) was layered on 2-mL columns of 45% Isolate (Irvine Scientific, Santa Ana, CA, USA) with or without 0.25% trypsin (trypsin-exposed and control, respectively), which overlaid 2-mL columns of 90% Isolate with or without 10  $\mu\text{g/mL}$  soy-based trypsin inactivator (Sigma, St. Louis, MO, USA) and centrifuged (700g for 30 min). The layering of multiple density gradients is facilitated by a novel polypropylene tube insert, which also prevents contamination when extracting treated sperm (USA and international patents pending). Pellets were washed and then incubated at room temperature. Sperm were examined (motility and supravital staining) at 0, 2, 24, and 48 h post-treatment and the results evaluated using Wilcoxon Signed Rank and Rank Sum tests. Exp. 2: A cytopathic cell (MT-2) assay was conducted (6 replicates) to determine the effect of trypsin (1-min exposure) on HIV-1 RNA infectivity. Viral replication was assessed by syncytium formation and p24 antigen production. Exp. 3: Two pools of fresh human semen ( $N1 = 3$  and  $N2 = 8$  donors) were inoculated (1:1) with  $1 \times 10^8$  copies/mL of cultured HIV-1 RNA, and one pool ( $N2$ ) was inoculated (1:1) with plasma collected from patients infected with either Hepatitis B DNA (HBV) or C (HCV) RNA viruses; spiked and non-spiked aliquots were processed as in Exp. 1. Treated sperm pellets were analyzed for HIV-1 or HBV and HCV concentrations by the Bayer Versant branched DNA (bDNA; version 3.0) and/or the Roche Amplicor quantitative RT-PCR (1.5 ultrasensitive) assays at Toga Laboratories (Pty), Ltd. (Edenvale, South Africa). As a result of Exp. 1, there was significantly ( $P < 0.05$ ) lower motility (but not supravital staining) between trypsin-treated and control sperm at 0 h (58.0 vs. 69.3%) and 2 h (54.7 vs. 62.9%) post-washing; however, no differences were noted after 24 h ( $P > 0.05$ ). In Exp. 2, trypsin exposure affected HIV-1 RNA infectivity negatively as compared to controls in terms of MT-2 cell syncytium formation and p24 antigen production. Results of the bDNA and/or RT-PCR assays in Exp. 3 indicated that the procedure effectively reduced HIV-1, HBV, and HCV viral copies in the spiked semen samples to undetectable levels or levels below clinical relevance. In conclusion, the novel trypsin density gradient procedure was effective for removing HIV-1, HBV and HCV from spiked semen without markedly affecting sperm survival. Extrapolation of these results to natural infections may be unfounded for viruses (e.g. HBV) that are thought to integrate into sperm chromatin.

# 186 A NOVEL METHOD FOR ELIMINATING PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS FROM BOAR SEMEN AND ITS EFFECTS ON EMBRYO DEVELOPMENT

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Porcine reproductive and respiratory syndrome virus (PRRSv) is known to cause venereal transmission of the disease via natural or artificial breeding and this constitutes a significant risk to AI programs in modern swine production. The objectives of this study were to determine the effectiveness of a novel density gradient centrifugation method incorporating trypsin to eliminate PRRSv from infected semen and to evaluate its effects on sperm viability and embryo development. Exp. 1: To assess the efficacy of the procedure on eliminating PRRSv, semen was collected from 21 infected boars. Concentrated sperm (1 mL) was layered on three Percoll (Sigma, St. Louis, MO, USA) density gradient columns, top to bottom: 1 mL 30%, 2 mL 45% with or without 0.25% trypsin (trypsin-treated and control, respectively), and 2 mL 90% with or without 10  $\mu\text{g/mL}$  soy-based trypsin inhibitor (Sigma), and centrifuged (700g for 30 min). Trypsin-treated and control sperm were submitted for RT-PCR analysis pre- and post-treatment. Exp. 2: To evaluate the effect on sperm quality, semen samples ( $n = 10$ ) were collected from non-infected boars and processed as described in Exp. 1. Sperm motility, viability, and acrosomal integrity were evaluated at 0 and 2 h post-treatment. Exp. 3.1: To assess the *in vitro* fertilizing capability of trypsin-treated sperm, *in vitro*-matured porcine oocytes ( $n = 64$ ) were inseminated, and cleavage (48 h post-insemination (PI)) and blastulation (144 h PI) rates were compared to those of oocytes ( $n = 63$ ) inseminated with control sperm. Exp. 3.2: Trypsin-treated or control sperm ( $3 \times 10^9/\text{dose}$ ) were used to AI sows ( $n = 10$ ). *In vivo*-generated embryos were surgically recovered 4–6 d post-AI, and embryo number, stage, and morphological quality were recorded. Data were analyzed using ANOVA and differences were considered significant at  $P < 0.05$ . Sperm quality parameters are expressed as means  $\pm$  SEM. Results showed that the procedure (with and without trypsin) was effective for eliminating PRRSv from infected boar semen. There were no differences at 0 or 2 h post-treatment between the control and the trypsin-treated boar sperm in motility ( $76 \pm 4.9$  and  $56 \pm 8.7$  vs.  $75 \pm 4.4$  and  $48 \pm 8.3\%$ , respectively), viability ( $87 \pm 2.6$  and  $75 \pm 6.2$  vs.  $81 \pm 3.2$  and  $80 \pm 3.7\%$ , respectively), and acrosomal integrity ( $96 \pm 2.7$  and  $98.8 \pm 0.8$  vs.  $98 \pm 1.3$  and  $99 \pm 0.4\%$ , respectively). There was no difference between the control and trypsin-treated sperm used for IVF on cleavage (82 vs. 89%, respectively) and blastulation (20 vs. 32%, respectively) rates. There were significantly more transferable-quality embryos recovered from sows inseminated with trypsin-treated as compared to control sperm: 54/63 (85.7%) vs. 35/72 (48.6%), respectively. In conclusion, the novel trypsin gradient treatment was effective in eliminating PRRSv without detrimentally affecting sperm quality and has the potential to increase the numbers of transferable-quality embryos produced.

## 187 ANALYZING DISEASE TRANSMISSION RISKS FROM ABATTOIR-DERIVED *IN VITRO*-PRODUCED BOVINE EMBRYOS

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While thousands of *in vitro*-produced (IVP) bovine embryos have been transferred commercially with no reports of disease transmission, such risks must be considered. Due to differences in their zonae pellucidae, the disease risks with IVP embryos are known to be higher than with *in vivo*-derived embryos. Possible sources of infection include the oocytes, spermatozoa, serum, and co-culture cells. The Terrestrial Animal Health Code of the Office International des Epizooties (OIE, 2003) stipulates that disease risk management should meet standards set by the World Trade Organization. These standards include subjecting the IVP procedures to quantitative risk assessment to evaluate disease transmission risk. The purpose of the present work was to measure the risks of transmitting disease with IVP embryos obtained from abattoir-derived tissues. A simulation model was developed using Microsoft Excel spreadsheets with the Palisade @RISK (London, UK) software program. The model incorporates probability distributions, the shapes of which reflect the random nature of some of the data (e.g. fluid volumes in cultures and washes) and the conjectural nature of some of the scientific information (e.g. on disease agents). The model is adaptable so that, when accurate data or information become available, variability estimates and degrees of uncertainty can be replaced with fixed values. The model assumes: (1) the IVP method is as described in the IETS Manual (1998); (2) there are five possible sources of infection; donor cow, donor bull, fetal calf serum, bovine serum albumin, and co-culture cells; (3) the disease agents can survive and/or proliferate during *in vitro* maturation, fertilization and culture; (4) fluid volumes in cultures and washes follow “known” normal distributions; (5) uncertainties in current knowledge of IVP embryos and disease agents can be taken into account by use of appropriate probability distributions; (6) different methods of *in vitro* fertilization do not affect the level of risk; and (7) different methods of *in vitro* culture can affect the level of risk. The model as constructed fits comfortably into a single workbook with one worksheet allocated for the model itself and another serving to store data on diseases of interest. Data on oocytes, blastocyst numbers, etc., and on media and wash fluid volumes are held within the model while information relating to particular diseases can be selected from a drop-down list at the top of the first worksheet. The relevant data stored in the database are then retrieved and used for modelling, using Monte Carlo simulation. The model estimates the final titer of the disease agent in IVP embryos and the probability of at least one infective transmission to a recipient, expressed as distributions.

### Exotic Species

## 188 DIRECT-THAW TRANS-CERVICAL TRANSFER OF RED DEER FROZEN *IN VITRO* BLASTOCYSTS CAN RESULT IN PREGNANCIES

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The seasonal demand for farmed venison in New Zealand has necessitated the concentration of red deer breeding into the first month of the four-month breeding season. Because of this constraint it is difficult to obtain enough *in vitro*-produced blastocysts for transfer. Successful cryopreservation would enable embryos produced and stored throughout the breeding season to be available for transfer the following year. *In vitro* red deer calves have been successfully produced after trans-cervical transfers in a limited number of red deer (Berg DK *et al.* 2004 Reprod. Fert. Dev. 16, 201 abst). We determined the viability of frozen blastocysts following trans-cervical transfer to recipient hinds using the direct-thaw method. In two replications, abattoir derived red deer COCs were selected and matured *in vitro* (Berg DK *et al.* 2002 Ani. Reprod. Sci. 70, 85–98). Oocytes were randomly divided into two groups and fertilized with either red deer sperm using IVF-Deer SOF (DSOF), or wapiti sperm using IVF-SOF. All presumptive zygotes were cultured for 6 days in DSOF (Beaumont SE *et al.* 2004 Reprod. Fert. Dev. 16, 268 abst). Cleavage was recorded on Day 4 and embryos were evaluated on Day 7. Grade 1 and 2 blastocysts were selected and equilibrated in 1.5 M ethylene glycol with 0.1 M sucrose, frozen from –5 to –38°C at a rate of 0.3°C per min and plunged into liquid nitrogen. Twenty synchronized farmed deer hinds (13 red deer to receive red deer blastocysts, and 7 F1 wapiti/red hybrids to receive F1 blastocysts) were prepared for transfer (Berg DK *et al.* 2003 Theriogenology 59, 189–205). Only Grade 1 blastocysts were selected for transfer. Straws were thawed for 5 s in air, immersed in a 30°C water bath for 20 s, directly diluted, and loaded into cattle transfer pistolettes. Each embryo was deposited in the uterine horn. A modified pistolette, fitted with a Mariensee tip (Minitüb, 84184 Tiefenbach, Germany) was used to dilate difficult cervixes ( $n = 4$ ). Pregnancies were confirmed by ultrasonography on Day 35. Results were evaluated using chi-square analysis. Embryo cleavage rates ranged from 74 to 85% and were not different between the two sires. Blastocyst development rates (from cleaved zygotes) were similar for both sires; wapiti 15% (43/279) and red deer 14% (34/246). A total of 24 wapiti/red hybrid and 17 red deer blastocysts were frozen. Eighteen of 20 hinds (90%) received embryos, 11/13 red deer receiving red deer embryos and 7/7 F1 wapiti/red hybrids receiving F1 wapiti/red hybrid embryos. The cervixes of two red deer hinds were impenetrable. Pregnancy rates were not different between the 2 groups of recipients, with 29% (2/7) of the wapiti hybrids and 45% (5/11) of red deer confirmed pregnant. These preliminary results demonstrate, for the first time, that farmed deer pregnancies can be established from frozen *in vitro*-produced embryos after direct-thaw and trans-cervical transfer to synchronized hinds.