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 cervices ($n = 4$). 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 cervices 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.

189 PRODUCTIVE AND REPRODUCTIVE PROFILE OF HOLSTEINS KEPT IN BALOCHISTAN PROVINCE, PAKISTAN

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Holstein-Friesian has been rated the number one cattle in the world as far as milk production is concerned. The productive potential is excellent in European countries. Animals of the same breed were imported to meet the milk demand of developing countries such as Pakistan. One thousand cattle from Denmark were imported in 1977–78 by the Balochistan government, and were kept at government dairy farms at seven locations (*viz*., Kalat, Khuzdar, Loralai, Mastung, Pishin, Quetta, and Zhob). The aim of this study was to determine the productive [age at first calving (AFC), birth weight (BWT), lactation length or milk yield (MY), milk yield per lactation (MYL), days in milk (DIM), and culling and mortality (C&L)] and reproductive potential [service period (SP), age at first service (AFS), gestation period (GP), and calving interval (CI)] of these imported animals while kept under the local conditions of Balochistan. Effect of localities with respect to the environmental data was also explored. Overall average values for AFS were 315 to 986 days with an average of 660.42 ± 12.42, GP 275 to 299 days with an average of 280.62 ± 0.25, and AFC 604 to 1299 days with an average of 944.08 ± 12.71. BWT ranged from 15 to 68 kg with an average of 30.12 ± 0.15, MY from 1471 to 7035 liters with an average of 3731.26 ± 40.52, DIM from 180 to 728 days with an average of 313.56 ± 3.83, and SP ranged from 29 to 886 days with an average of 240 ± 9.61, DP averaged 59.15 ± 20.61 days and CI ranged from 301 to 922 days with an average of 451.10 ± 5.55. The effects of year, season, age, and location were studied and the results revealed that the year influenced the AFS, AFC, BWT, and SP ($P < 0.01$); GP ($P < 0.05$) but not DIM and CI ($P > 0.05$). No effect of season on AFS, AFC, DIM, MY, SP or CI ($P > 0.05$) was observed, but GP and BWT ($P < 0.05$) were affected by season. Age influenced the GP and SP ($P < 0.05$) but not BWT, DIM, MY, and CI ($P > 0.05$). No effect of sex of the calves, type of birth, and calving number on GP ($P > 0.05$) was seen. Location of the farm did effect AFS, AFC, BWT, and MY ($P < 0.01$); CI ($P < 0.05$); but not GP, DIM, and SP ($P > 0.05$). In conclusion, differences in environment and seasonality had a variable impact on production parameters in Holstein-Friesian calves.

190 A PROCEDURE TO OBTAIN FIBROBLASTS FROM WILD ANIMALS

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The number of wild animals threatened or at risk of extinction constantly increases and, as a result, biodiversity decreases. Therefore, it is necessary to set in motion proceedings to preserve the genetic variability and to overcome factors leading to this situation. Banks of cryopreserved cells, such as skin fibroblasts, from wild animals could be used both for genetic studies and as a cell source for technologies able to reproduce individuals. In this study we report that a single basic protocol provides a method for obtaining, culturing, and cryopreserving skin fibroblasts from a wide range of wild animals. Skin biopsies were taken in Cantabria (Spain) from live, anesthetized brown bear (*Ursus arctos*, $n = 4$), and in Asturias (Spain) from carcasses of accidentally killed individuals from the following species: grey wolf (*Canis lupus*, $n = 1$), red fox (*Vulpes vulpes*, $n = 1$), eurasian badger (*Meles meles*, $n = 1$), ermine (*Mustela erminea*, $n = 1$), fallow deer (*Dama dama*, $n = 7$), pyrenean chamois (*Rupicapra pyrenaica*, $n = 1$), western roe deer (*Capreolus capreolus*, $n = 1$), wild boar (*Sus scrofa*, $n = 1$), striped dolphin (*Stenella coeruleoalba*, $n = 1$) and one avian species, capercaillie (*Tetrao urogallus*, $n = 1$). Once obtained and transported to the laboratory, all samples were processed identically. Tissue samples were manually cut into small pieces and enzymatically digested with 0.05% trypsin-EDTA (Sigma-Aldrich, Madrid, Spain) for 30 min, and then with collagenase (300 units/mL) (Sigma) for 14 h at 38°C. Disaggregated cells were centrifuged at 1600 rpm for 10 min and the pellet was diluted with D-MEM (Sigma) containing 10% FBS. Cells were counted and a minimum of 3×10^5 cells were placed in a 25 cm^2 flask for culture under 5% CO_2 in air and high humidity at 37°C (red fox, badger, ermine, western roe deer) or 38°C (brown bear, grey wolf, fallow deer, pyrenean chamois, wild boar, striped dolphin, capercaillie). Confluent fibroblast monolayers were obtained after 5–7 days in culture except for the striped dolphin whose sample was contaminated. Two to three passages were performed using 75 cm^2 flasks before freezing fibroblasts in D-MEM containing 10% DMSO (Sigma) and 10% FBS. Total cell yield from a confluent flask was $5–11 \times 10^6$ cells, depending on the animal species. Fibroblasts recovered from all thawed samples grew to confluence, and karyotypes were performed at this step. It was possible to obtain, culture, freeze, and thaw skin fibroblasts successfully from all the above-mentioned land species. Although more research is needed, preliminary data suggest that the above-described procedure can be applied to mammals and birds, thus helping to preserve genetic material of wild animals.

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191 TIMING OF OVULATION IN THE GONADOTROPHIN-STIMULATED SOUTHERN HAIRY-NOSED WOMBAT, *LASIORHINUS LATIFRONS*

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The southern hairy-nosed wombat (SHW), *Lasiorhinus latifrons*, is a model species in which to develop assisted breeding techniques for the endangered northern hairy-nosed wombat, *Lasiorhinus krefftii*. We recently showed that anoestrus SHW respond to eutherian gonadotrophins by production of multiple ovarian follicles, but ovulation had not occurred at the time of examination 24 h post-LH (Druery GV *et al*. 2003 Theriogenology 59, 391 abst). This study investigated the timing of ovulation in six anoestrus captive adult female SHW ($n = 3$ per group) after ovarian superstimulation using porcine FSH (200 mg total, Folltropin-V, Bioniche, Belleville, Ontario, Canada) administered s.c. at 12-h intervals over 7 days. Ovulation was triggered by a single s.c. dose of porcine LH (25 mg Lutropin-V, Bioniche) 12 h after the final FSH injection. Superstimulatory response was determined by laparoscopy immediately after the final FSH injection on Day 7 prior to LH. Group 1 was re-examined at 33, 36, and 39 h post-LH, and Group 2 at 42, 45, and 48 h post-LH, for evidence of ovulations using laparoscopy and transabdominal ultrasonography. Laparoscopy on Day 7 revealed an ovarian follicular response in all six females, which coincided with the highest levels of estradiol. The reproductive tract also responded to the treatment (swollen fimbriae and enlarged, highly vascular uteri). Multiple follicles (range 16–31) up to 11 mm in diameter were observed in five females. One female had ovulated, as determined by the presence of corpora lutea. Transabdominal ultrasonographic imaging was unable to confirm the number of follicles in stimulated ovaries. Ovulation had commenced by 36 h post-LH, with the majority occurring 39–45 h post-LH. Ovulation was recorded as having occurred if a dark red, highly vascular crater on the surface of the newly formed corpus hemorrhagicum was observed. Increased circulating levels of progesterone were confirmed 9 days after the last laparoscopies. These results have important implications for the development of assisted reproductive technologies in the SHW: (1) transabdominal ultrasound imaging is ineffective for determining ovarian activity; (2) laparoscopy is a well-tolerated, repeatable minor surgical procedure that can be used for intrauterine AI in this species in which nonsurgical AI is unlikely to succeed (Paris DBBP *et al*. 2003 Theriogenology 59, 401 abst); and (3) knowledge of the timing of ovulation will enable insemination of spermatozoa into the uterus prior to ovulation.

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192 PROBLEMS USING JC-1 TO ASSESS MITOCHONDRIAL STATUS IN BROWN BEAR (*URSUS ARCTOS*) SEMEN

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Brown bear is a highly endangered species in Spain and could benefit from biological resource banking. Currently, we are studying several reproductive aspects in order to aquire the knowledge for establishment of a germplasm bank for this species. One of our objectives is to develop adequate protocols for the evaluation of bear sperm before and after cryopreservation. We have used the fluorescent probe JC-1 protocol, which differentially stains mitochondria, according to its activity (Garner DL *et al*. 1997 Biol. Reprod. 57, 1401–1406). Here we describe one problem that arose using this staining for evaluation of extended bear semen. We electroejaculated 13 adult brown bears (*Ursus arctos*) (206–311 kg) housed in a half-freedom regime in the Cabarceno Park (Cantabria, Spain). Anesthesia was performed with tiletamine + zolazepan (Zoletil 100®, 7 mg/kg; Virbac, Spain), and ketamine (Imalgene 1000®, 2 mg/kg; Mericl, Sain). We used an electroejaculator (PT Electronics®; Boring, OR, USA) with a 3-electrode transrectal probe (26 mm in diameter, 320 mm long). Ejaculation occurred at 10 V/250 mA. Samples were extended (prepared in our laboratory, Anel L *et al*. 2003 Theriogenology 60, 1293–1308; M3 modified) and cooled to 5°C for 70 min (pre-freezing protocol). We analyzed individual (MI) and progressive (MP) motility by means of an automated motility analyzer (Hamilton Thorne Biosciences, Inc., Beverly, MA, USA), using a phase contrast microscope (Nikon, ×10). Mitochondrial status was analyzed after diluting the sample 1:100 with buffered medium (20 mM HEPES, 153 mM NaCl, 2.5 mM KOH, 10 mM glucose; Sigma, Madrid, Spain) and adding JC-1 (6.8 μM final; Molecular Probes, The Netherlands). After 30 min at 37°C, 100 cells were counted with an epifluorescence microscope (Nikon, ×400), determining the percentage of sperm with orange-stained (active) mitochondria. We analyzed a total of 55 samples in three different models: fresh, pre-freezing, and thawed. We divided the samples into successful JC-1 staining (valids: V) or failed JC-1 staining (not valid: NV) (depending on the aspect of the stained cells). In not-valid samples we observed a greenish background, with almost no fluorescent spermatozoa. These observations were consistent in a given sample, giving the same V or NV result when we repeated the staining. In fresh and thawed groups there were no NV samples, but in the pre-freezing group there were 40 NV samples (73%). We calculated Pearson correlations (SAS; SAS Institute, Inc., Cary, NC, USA) between percent JC-1 orange population and MI and MP in fresh ($r = 0.40$ and 0.33; $P < 0.001$), thawed ($r = 0.61$ and 0.43; $P < 0.001$) and pre-freezing samples ($r = -0.11$ and -0.24; $P > 0.05$), all respectively. When pre-freezing samples were split between V and NV, the former had good correlations ($r = 0.74$ and 0.49; $P < 0.05$), and NV still did not ($r = -0.17$, -0.27; $P > 0.05$). We conclude that JC-1 staining is not reliable for the pre-freezing analysis of bear sperm, at least under the conditions described here. This could be due to the interaction of the extender or the refrigeration treatment with the sperm. However, this problem did not occur in the analysis of fresh and thawed samples. Nevertheless, it may be advisable to test other mitochondrial probes for analyzing this kind of samples.

193 TESTIS TISSUE XENOGRAFTING TO PRESERVE GERM CELLS FROM A CLONED BANTENG CALF

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In April 2003, two banteng (*Bos javonicus*) calves were born after heterologous nuclear transfer of donor cells from a genetically valuable individual frozen in 1978. One of the cloned banteng calves died at one week of age. The calf was found to have one scrotal and one abdominally cryptorchid testis. In an attempt to preserve male germ cells from this valuable animal, parts of each testis were shipped on ice to the University of Pennsylvania for xenografting. Grafting of testis tissue from immature domestic animals and monkeys under the back skin of immunodeficient mice can result in complete spermatogenesis, albeit with different levels of efficiency in different species. The objective of this experiment was to investigate if grafting of immature banteng testis tissue would result in spermatogenesis in a mouse host. Small fragments of tissue (about 1 mm, 3 each) from both testes were grafted under the back skin (4 pieces of scrotal testis on the right side and 4 pieces of retained testis on the left side) of 6 castrated male immunodeficient mice. Histological examination of the testis xenografts was performed 3, 6, 9, 12, and 15 months after transplantation. Weight of the seminal vesicles in the host mouse was recorded as an indicator of bioactive testosterone produced by the xenografts. At the time of grafting, both testes contained seminiferous cords with immature Sertoli cells and gonocytes. At 3, 6, and 9 months after grafting, pachytene spermatocytes were present in the xenografts of the scrotal testis whereas no germ cell differentiation was observed in grafts from the retained testis. However, spermatogenesis in grafts of the scrotal testis did not proceed further through meiosis in grafts analyzed at 12 and 15 months after grafting, with pachytene spermatocytes still the most advanced germ cell type present in grafts recovered 15 months after grafting. The weight of the seminal vesicles in the castrated host mice was restored to pre-castration values showing that xenografts were releasing bioactive testosterone. These results indicate that banteng spermatogenesis was initiated in the mouse host but became arrested at meiosis as observed previously in xenografts of immature bovine or equine testis. Therefore, haploid germ cells could not be recovered. This represents the first example of trying to preserve fertility from a rare, valuable newborn animal by testis tissue xenografting. While xenografting presents a previously unavailable option for preservation of male germ cells from immature individuals, the efficiency of sperm production in testis xenografts appears to be variable and has to be determined empirically for different donor species.

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194 BIRTH OF KITS AFTER STORAGE IN CULTURE AND TRANSFER OF *IN VIVO* EMBRYOS IN THE FARMED EUROPEAN POLECAT (*MUSTELA PUTORIUS*)

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The effect of *in vitro* culture on viability of pre-implantation stage embryos in the farmed European polecat was studied, aimed at developing assisted reproductive technology for conservation of endangered mustelids, particularly the European mink (*Mustela lutreola*). Embryo storage in culture would enable embryo recovery and transfer in different locations. Ferret (*Mustela putorius furo*) kits have been produced from embryos that were cultured for 3 days in serum-containing medium (Li *et al*. 2001 Reproduction 122, 611–618). In our earlier studies, polecat embryos were maintained for 24 h in culture conditions (Lindeberg *et al*. 2003 Theriogenology 60, 965–970). Fourteen estrous donors were kept in the same cage with a fertile male overnight and sacrificed 3 days after the start of mating for recovery of embryos from the oviducts. Embryos were flushed with Emcare™ Complete ultra flushing medium (ICPBio, Auckland, New Zealand), washed twice in it, washed once in Emcare™ embryo holding solution and transported in the holding solution at room temperature for 1 h to the laboratory. Embryos of seven donors were pooled and cultured in 30-μL drops of TCM199 + glutamax I (GIBCO™) supplemented with fatty acid-free albumin (FAFBSA, Sigma-Aldrech, Helsinki, Finland) under a cover of paraffin oil (Medicult) for 3 days in a humidified atmosphere (39°C) and in 5% of O_2. At the end of the culture, the embryos were evaluated and the ones that had developed at least to morula stage were chosen for transfers. The selected embryos were transported at room temperature in Emcare™ embryo holding solution for 1 h to the farm where they were surgically transferred under general anesthesia into seven recipients. The recipients had been mated the same way as the donors but with vasectomized males either on the same day as the donors (the first set: 7 donors, 3 recipients) or one day later than the donors (the second set: 7 donors, 4 recipients). Five embryos were cultured a total of 6 days to the blastocyst stage and stained for a count of cell numbers. A total number of 169 one- to 16-cell-stage embryos were recovered. At the end of the 3-day culture period, a total of 139 (139/169, 82%) had developed to morula (56.6%), compact morula (9.8%), early blastocyst (30.3%), or blastocyst stage (3.3%). Of these 139 embryos, a total of 102 were surgically transferred. Five of the 7 recipients delivered one to 5 kits each 43 to 45 days after the mating. Altogether 21 kits were born and the success rate was 21% (21 kits/102 transferred embryos). Cell numbers of the five Day 6 blastocysts varied from 130 to 430. In conclusion, this preliminary trial confirms that polecat embryos can be stored in culture for 3 days. In this study polecat embryos were cultured in 5% oxygen and without addition of serum which resulted in considerably better cell numbers for Day 6 blastocysts than in our earlier studies (90 to 165 cells; Lindeberg *et al*. 2003 Theriogenology 60, 965–970).

195 CHANGES OF BLESBOK AND BLUE WILDEBEEST EPIDIDYMAL SPERM AFTER INCUBATION AT 37°C

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Postmortem recovery of epididymal spermatozoa and their preservation in Biological Resource Banks is a convenient source of germplasm, providing a possible future conservation resource for selected endangered wildlife species. It is necessary to gain knowledge of the biology of the gametes of the different species, in order to define effective protocols for cryopreservation and future assisted reproductive technology application. A pilot study on the changes in blue wildebeest (*Connochaetes taurinus*) and blesbok (*Damaliscus dorcas phillipsi*) epididymal sperm was carried out in order to provide some insight into the effects of incubation at 37°C. Chemicals were aquired from Sigma (South Africa), except JC-1 (Molecular Probes, Leiden, The Netherlands). Sperm was obtained by flushing the vas deferens and cauda epididymis of 6 adult blue wildebeests and 4 adult blesbok after the breeding season using 1 mL of Biladyl (fraction A; Minitüb, Tiefenbach, Germany). Cells were washed and resuspended in buffered medium (20 mM HEPES, 197 mM NaCl, 10 mM glucose, 2.5 mM KOH). Part of each sample was analyzed and part was incubated for 1 h at 37°C, and then analyzed. Analysis consisted of: motility (% of motile sperm, TM; and % of linear sperm, LM), vitality (fluorescent dye propidium iodide, 7 μM; % of unstained cells noted after 10 min at RT: vital, VIT), mitochondrial status (fluorescent dye JC-1, 7.5 μM; % of cells with orange midpiece noted after 30 min at 37°C: active mitochondria, MIT), and induction of acrosome reaction (15 min at 37°C in buffered medium complemented with 3 mM CaCl; % of intact acrosomes noted in control: splits no ionophore, ACR, and test: splits 1 μM calcimycin, ION). Samples were assessed using phase contrast microscopy (×400; ×200 for motility). Results are showed in Table 1. No significant differences (Wilcoxon Rank Sign test) were detected, possibly due to the low number of samples. However, LM appeared to decrease after incubation. Incubaton may increase the sensitivity of blue wildebeest sperm to ionophore (ION). Motility was least for blesbok, and the decrease of LM after incubation was more apparent. This treatment may induce different physiologycal changes between the species (different LM variation). The rest of the parameters suggest that the treatment did not induce extensive cell damage. Further research must be carried out to confirm these findings.

Table 1. Median values for the analyzed parameters

Species	Analysis	TM	LM	VIT	MIT	ACR	ION
Blesbok	0 h	72.5	37.5	83.5	90	–	–
	1 h	62.5	15	77.5	88	–	–
Blue wildebeest	0 h	87.5	60	85.5	90.5	81	71
	1 h	85	47.5	81	88	86	63
	After	85	47.5	81	88	86	63

0 h and 1 h refer to incubation at 37°; – = could not be assessed.

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196 *IN VITRO* DEVELOPMENT OF RECONSTRUCTED WATER BUFFALO (*BUBALUS BUBALIS*) OOCYTES AFTER FETAL SKIN FIBROBLAST CELL NUCLEAR TRANSFER

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The present study was undertaken to explore the feasibility of using buffalo fetal skin fibroblasts as donor nuclei and to determine the developmental competence of embryos following transfer of these nuclei to *in vitro*-matured enucleated buffalo oocytes. Skin cells were isolated from 1–2-month-old fetuses, obtained from an abattoir, by enzymatic digestion (0.5% w/v trypsin + 0.05% w/v collagenase in Dulbecco's PBS) for 15–20 min. The cells were washed four times with Dulbecco's PBS and then once with RPMI-1640 medium + 10% FBS by centrifugation at 600*g*. The cells were then cultured in the same medium in a CO_2 incubator (5% CO_2 in air) at 38.5°C for 2–3 days. COCs collected from slaughterhouse buffalo ovaries were subjected to IVM in the IVM medium (TCM-199 + 1 μg mL^{-1} E-β + 5 μg mL^{-1} FSH-P + 10 μg mL^{-1} LH + 10% FBS) for 22–24 h in a CO_2 incubator (5% CO_2 in air) at 38.5°C. Oocytes were denuded with 0.1% trypsin followed by repeated pipetting and then enucleated by aspirating the first polar body and 10–15% of nearby cytoplasm with a micromanipulator. Two different types of donor cells (growing cells and those arrested with cytochalasin-B) were used for reconstruction of oocytes. The reconstructs were electrofused and incubated in the activation medium (TCM-199 + 8 μg mL^{-1} cytochalasin-B + 10% FBS) for 4 h. These were then cultured in IVC medium (TCM-199 + 10% FBS) in a CO_2 incubator (5% CO_2 in air) at 38.5°C for 48 h. Next, the cleaved embryos were co-cultured with buffalo oviductal cells in embryo development medium. Out of 119 denuded matured oocytes which were enucleated and reconstructed with growing cells, 78 (65.5%) were electrofused, activated and cultured, out of which 4 (5.1%) reconstructs cleaved and developed to the 2-cell stage, 3 (3.8%) reached the 4-cell stage, and 1 (1.3%) reached the 8-cell stage. In the synchronized group, out of 100 denuded matured oocytes which were reconstructed with cytochalasin-B blocked cells, 40 (40%) were electrofused, activated, and cultured, out of which 4 (10%) developed to the 2-cell stage, 3 (7.5%) to the 4-cell stage, 2 (5.0%) to early morula stage, and 1 (2.5%) to blastocyst stage. These results suggest that buffalo fetal skin fibroblasts could be used as donor nuclei for the production of buffalo embryos after nuclear transfer to enucleated *in vitro*-matured buffalo oocytes.

197 HYSTRICOMORPH SUBPLACENTA: THE COMPARATIVE ASPECTS

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Placentae of four hystricimorph rodents – capybara (*Hydrochaerus hydrochaeris*), agouti (*Dasyprocta aguti*), paca (*Agouti paca*), and rock cavy (*Kerodon rupestris*) – were examined by conventional histology, transmission electron microscopy, and scanning electron microscopy. In all of these animals, the subplacenta comprised cytotrophoblasts, supported on lamellae of allantoic mesoderm, and syncytiotrophoblasts. The subplacenta of paca, agouti, and rock cavy had a structure similar to that of capybara, although it was not clearly separated from the main placenta by a layer of mesenchyme. The subplacenta of agouti was examined by conventional histology and found to have a structure similar to that in capybara and paca. The cytotrophoblasts were separated by true extracellular spaces; these cells were characterized by the presence of numerous mitochondria, a large-size nucleus relative to the cytoplasm, and absence of glycogen and lipid (PAS-negative). Also, the morphology of the syncytiotrophoblasts was similar in all of these animals; these cells were PAS-positive, and were characterized by the presence of numerous spherical electron-dense granules distributed throughout the cytoplasm, presence of large accumulations of glycogen, few mitochondria, golgi apparatus and granular ER. In addition, the syncytium contained lacunae lined by microvilli, observed only by transmission electron microscopy. The subplacenta is supplied by a single fetal artery. The vessels within the subplacenta pursue a tortuous course with dilatations and constrictions as in an endocrine gland. In mid-to-late gestation, the subplacenta is supplied largely by fetal vessels. A large branch of the umbilical artery follows the central band of fetal mesenchyme to the base of the main placenta and then branches to supply the subplacenta. The subplacental vessels pursue a tortuous course with dilatations and constrictions as in an endocrine gland such as the ovary. A few branches of the maternal artery supply the subplacenta, at least in capybara and paca, where they can be demonstrated by latex injection.

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198 DISTRIBUTION OF SPERMATOZOA AND COPULATORY PLUG IN RELATION TO THE TIME OF MATING AND OVULATION IN THE FEMALE TAMMAR WALLABY (*MACROPUS EUGENII*)

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In the monovular macropodid marsupial, the tammar wallaby (*Macropus eugenii*), the cervices are the primary selective barrier to spermatozoa, resulting in differential transport to the non-gravid uterus where a sperm reservoir is established (Tyndale-Biscoe CH and Rodger JC 1978 J. Reprod. Fertil. 52, 37–43). However, due to limited sample size, the dynamics of sperm transport could not be thoroughly examined. In this study, the distribution of spermatozoa, the size of the copulatory plug in the reproductive tract at various times after mating, and the timing of ovulation were characterized in 28 naturally mated female tammars. After the first postpartum (p.p.) mating, adult females were isolated and their reproductive tracts dissected at 0.5, 6, 18, 36, and 40 h post-coitum (p.c.). Each tract was ligated into 13 major anatomical sections, and spermatozoa and eggs were recovered by flushing. Mating occurred 21.7 ± 2.5 h p.p. (mean $\pm$ SEM; $n = 20$) in these animals that were checked frequently and lasted 7.8 ± 0.7 min ($n = 15$). Within 0.5 h after a single mating ($n = 5$) the tract contained $2.6 \pm 1.0 \times 10^7$ spermatozoa and 21.6 ± 8.8 g of copulatory plug, 96 and 70% of which was lost within 6 h p.c., respectively. Spermatozoa reached the uterus, isthmus, and ampulla of the oviduct ipsilateral to the developing follicle within 0.5, 6, and 18 h p.c. respectively, and a uterine population of $2.6 \pm 1.2 \times 10^4$ spermatozoa ($n = 24$) was maintained for over 40 h (ANOVA, $P > 0.05$). Sperm numbers were reduced at the cervix (up to 57-fold) and utero-tubule junction (8-fold), and only 1 in ~7600 ejaculated spermatozoa ($3.4 \pm 0.9 \times 10^3$; $n = 14$) reached the oviduct on the side of ovulation. Although sperm numbers were reduced in the gravid uterus ($n = 24$), differential transport of spermatozoa was not observed (ANOVA, $P > 0.05$). Ovulation and recovery of sperm-covered eggs from the isthmus of the oviduct occurred 36–41 h p.c. (49–72 h p.p.) ($n = 8$). Like many eutherian mammals, but in contrast to polyovular dasyurid and didelphid marsupials, the tammar ejaculates large numbers of spermatozoa, but transport is relatively inefficient and sperm storage in the tract before ovulation is limited.

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199 BASIC CHARACTERISTICS AND CRYOBANKING OF BARBARY SHEEP (*AMMOTRAGUS LERVIA*) SEMEN

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Barbary sheep (*Ammotragus lervia*) are considered vulnerable species by the World Conservation Union (IUCN). The purpose of this study was to describe the basic characteristics of fresh semen, test the efficacy of commercial extender Triladyl, and collect necessary data that may help

to create a frozen semen bank for the Barbary sheep in Spain. A total of 21 ejaculates were collected by rectal-probe electroejaculation from one dominant (D) and three minor (M) adult males housed in the Madrid Zoo. After ejaculation, semen volume, concentration, and mass motility were assessed. Remaining raw semen was diluted at 37°C with TRIS-based extender Triladyl (Minitüb, Tiefenbach, Germany) and 20% egg yolk to a final concentration of 200×10^6 sperm per mL. Diluted samples were kept at 5°C for 4 h and then loaded into 0.25-mL French straws, frozen at 5 cm above liquid nitrogen (LN_2) for 10 min and then plunged into LN_2. Samples were thawed in a water bath at 37°C for 30 s. Post-thaw semen survival was evaluated by sperm motility (%M), quality of movement (Q), normal acrosome status (%NAS), normal sperm morphology (%NOR), membrane integrity (hypo-osmotic test; %HOST), and sperm viability (eosin-nigrosin vital staining; %V), and were compared with the same parameters in the fresh semen. Data between D and M males were analyzed by one way ANOVA. Mean volume of ejaculates, total sperm concentration and mass motility of raw semen were respectively; 5.2 ± 1.56 mL, $2800.0 \pm 1290.5 \times 10^6$ and 3.4 ± 0.4 for the D male, and 3.5 ± 3.2 mL, $251.2 \pm 103.9 \times 10^6$, and 1.88 ± 1.4 for M males ($P < 0.05$). Remaining semen parameters evaluated in raw semen showed no differences between D and M males. However, post-thaw semen quality was significantly ($P < 0.05$) reduced in all analyzed parameters except %NAS and %NOR in M males groups as compared to the D male (Table 1). It can be concluded that Barbary sheep raw semen collected by electroejaculation is of sufficient quality to be used in an artificial insemination program and can be successfully frozen in commercially available Triladyl extender. However, the post-thaw viability of semen may considerably depend on the male reproductive status in the flock.

Table 1. Characteristics of fresh and cryopreserved Barbary sheep semen

	Fresh		Frozen-thawed	
	D	M	D	M
%M	84.4 ± 5.0	75.0 ± 12.9	65.0 ± 25.5^a	25.0 ± 19.1^b
Q	4.4 ± 0.2	4.4 ± 0.0	4.3 ± 0.3^a	2.8 ± 1.9^b
%NAS	83.8 ± 11.6	84.5 ± 6.8	71.0 ± 10.3	48.5 ± 34.6
%NOR	91.6 ± 6.7	94.2 ± 6.6	91.0 ± 4.6	91.2 ± 3.2
%HOST	57.8 ± 9.5	64.7 ± 17.7	39.0 ± 15.5^a	20.0 ± 12.9^b
%V	80.8 ± 6.4	82.2 ± 8.3	75.1 ± 8.0^a	40.7 ± 24.0^b

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

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200 IMMUNOHISTOCHEMICAL LOCALIZATION OF VASCULAR ENDOTHELIAL GROWTH FACTOR AND ITS RECEPTORS, Flt-1 AND KDR, IN THE COLLARED PECCARY (*TAYASSU TAJACU*) PLACENTA

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Vascular endothelial growth factor (VEGF) is a well-known angiogenic growth factor, which has previously been localized in epitheliochorial, endotheliochorial, and hemochorial placenta types. The present study was undertaken to study the localization of VEGF and its ligands during gestation in the placenta of the *Tayassu tajacu* (collared peccary), which belong to the Family Tayassuidae, the super family Suoidea, and the order Artiodactyla. The 90-cm long, 30-kg collared peccary is the smallest peccary and it has a white collar around the neck. The animals are very well adapted in all Brazilian territory reproducing during all seasons of the year; it has a gestational period of 144–148 days. The placenta is diffuse and mutually folded with areola-gland subunits for absorption of uterine gland secretions. In the peccary placenta the interhemal barrier is epitheliochorial and it is andeciduated. In the present study, the VEGF and its receptors VGFR-1 and VGFR-2 or Flt-1 and KDR, respectively were immunolocalized in the epitheliochorial collared peccary placenta. The animals were obtained from Sao Pedro Farm, Sao Paulo (Brazil) and from CEMAS, Rio Grande do Norte (Brazil). Six placentae of pregnant collared peccary (60, 90, and 110 days gestation) were prepared for immunohistochemistry by routine methods. VEGF, Flt-1, and KDR exhibited intense staining in the uterine epithelium, uterine glandular epithelium, and trophoblast. The endothelial cells and smooth muscle cells of the vessels in maternal and fetal compartments were also immunoreactive. In the pig placenta the VEGF-ligand-receptor system participates in placental regulations, and we have shown here that these factors occur in peccary placenta in a similar or comparable way.

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201 EFFECT OF HEPARIN AND CALCIUM IONOPHORE ON ACROSOME REACTION IN EPIDIDYMAL SPERMATOZOA OF DROMEDARY CAMEL (*CAMELUS DROMEDARIUS*)

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The present study was undertaken to evaluate the effect of heparin and calcium ionophore (Ca-I) on the induction of acrosome reaction in epididymal spermatozoa of dromedary camel. Cauda epididymides were isolated from testes of mature males immediately after slaughter at a local abattoir

and brought to laboratory in saline solution on ice (0–1°C). Spermatozoa were collected by aspirating the sperm-rich fluid from convoluted tubules beneath the ductus deferens with a sterile hypodermic needle attached to a 10-mL syringe containing 2–4 mL of TRIS-tes egg yolk diluent and stored overnight at 4°C. The sperm suspension was aliquoted into 3 tubes, washed twice with fertilization medium (TALP; Parrish *et al*. 1985 Theriogenology 24, 537); pellets were dissolved in 1 mL of fertilization medium supplemented with 10 μg/mL heparin or 10 μM/mL Ca-I or no additive (control). Aliquots (200 μL) were made in Eppendorf tubes and incubated at 38°C for 0.5, 1, 1.5, or 2 h. Sperm viability and acrosomal status was evaluated by a dual staining procedure (Didion *et al*. 1989 Gamete Research 22, 51–57). Briefly, spermatozoa were incubated with an equal volume of 0.2% trypan blue for 10 min and washed twice (centrifugation at 700*g* for 6 min) with fertilization medium. Smears were made on glass slides and dried quickly on a warm stage. Slides were stained with 10% giemsa stain for 40 min. They were rinsed under a stream of distilled water, air-dried, and covered with DPX mountant and coverslips. The experiment was replicated six times. Spermatozoa ($n > 200$/slide) were examined in randomly selected microscopic fields under a phase-contrast microscope at 1000×. Spermatozoa were classified as dead (stained blue in the post-acrosomal region), live non-acrosome-reacted (unstained post acrosomal region but light purple-dark pink acrosome) or live acrosome reacted (unstained acrosome and post-acrosomal region). Data were analyzed using Student's *t*-test and are presented as mean percent ± SEM. There was no difference in the proportion of live acrosome-reacted (LAR) and live non-acrosome-reacted (LNAR) spermatozoa in the heparin group compared to the control group (Table 1). Although Ca-I group had higher LAR sperm, the proportion of dead spermatozoa was high ($P < 0.05$). It may be concluded that heparin was not able to enhance the acrosome reaction, and Ca-I seems to be detrimental to the spermatozoa at this concentration. Further investigations are warranted to evaluate the appropriate concentrations and duration of exposure to both of these agents for induction of acrosomal reaction in spermatozoa of this species.

Table 1

Treatment	Time (h)	LAR	LNAR	Dead
Heparin (10 μg/mL)	0.5	8.8 ± 1.8	55.2 ± 1.9	36.0 ± 1.9
	1	10.4 ± 1.4	55.0 ± 4.4	34.6 ± 4.3
	1.5	9.8 ± 1.2	55.5 ± 3.7	34.7 ± 2.5
	2	9.4 ± 1.5	54.7 ± 8.1	35.9 ± 6.5
Calcium ionophore (10 μM/mL)	0.5	18.4 ± 4.7*	16.5 ± 3.8†	65.1 ± 5.7*
	1	16.7 ± 3.3*	5.4 ± 1.3†	77.9 ± 2.3*
	1.5	18.3 ± 3.0*	2.5 ± 1.5†	79.1 ± 1.7*
	2	12.8 ± 3.8	2.2 ± 1.3†	85.0 ± 3.6*
Control	0.5	7.3 ± 1.0	58.2 ± 4.3	34.5 ± 4.6
	1	8.7 ± 1.0	51.6 ± 4.9	39.7 ± 3.8
	1.5	10.7 ± 1.7	51.4 ± 4.4	37.8 ± 3.1
	2	9.5 ± 1.6	50.9 ± 6.2	39.6 ± 5.8

* Significantly higher; † significantly lower than other values in same column, Student's *t*-test ($P < 0.05$).

Folliculogenesis/Oogenesis

202 GENETIC INFLUENCE ON FOLLICULAR DEVELOPMENT IN CATTLE

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In cattle, the development of ovarian follicles >5 mm occurs in waves. During each estrus cycle, a cohort of 5–7 follicles develops either 2 or 3 times, with the last wave containing the ovulatory follicle. It is speculated that follicular wave number within estrus cycle may be genetically determined; however, no data have been published to support this hypothesis. The present study was designed to test this hypothesis by using genetically identical cloned cows and comparing their wave patterns among each other and to those of control animals by studying their ovarian follicular dynamics. Three 5-yr-old cloned cows, derived from somatic cells of a 13-yr-old Holstein dairy cow, were age- and lactational status-matched with contemporary cows produced by AI, and maintained under the same managemental conditions. Ovarian follicular dynamics of all the cows were determined using a real-time, B-mode, linear-array ultrasound scanner equipped with a 7.5 MHz transrectal transducer (Aloka Co., Tokyo, Japan). Ultrasound examinations were carried out every day for 101 days and covered 4 estrus cycles for each animal. For tracking the follicular dynamics, daily ultrasound observations were videotaped and individual follicle dimension and location were drawn. The estrus was determined by the visual observation of a growing follicle over a period of several days, reaching a maximum diameter of about 15 mm or more, and its subsequent disappearance on the following day. The ultrasound observations were corroborated with the visual and behavioral signs of estrus. The average length of estrus cycle in clones and controls was 23.08 ± 0.47 days and 22.67 ± 0.37 days (mean ± SEM), respectively, and were not significantly different. Out of the four estrus cycles studied, all clones showed 3 follicular waves in 3 cycles and 2 waves in one cycle. However, the wave pattern within each cycle did not match for the clones over all the cycles. In controls, one cow had 2 waves in all 4 cycles while the other two cows had both 3- as well as