



A024E Physiology of Reproduction in Male and Semen Technology

Vitrification of epididymal sperm from Iberian ibex (*Capra pyrenaica*)

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Keywords: cryopreservation, Iberian ibex, semen vitrification.

Vitrification, a process in which living cells undergo glasslike solidification, is a relatively new cryopreservation method that can successfully preserve the embryos, oocytes, and even the sperm of certain species. For sperm, at least in the species studied so far, kinetic vitrification would appear to be a better alternative. The simplicity of this sperm cryopreservation technique can be useful in field laboratories for wild species because requires less equipment, is much faster, simpler and cost-effective than conventional freezing. The aim of this work was to evaluate comparatively the effectivity of kinetic vitrification and conventional freezing of epididymal sperm from Iberian Ibex (*Capra pyrenaica*). Testes were obtained from mature ibexes that were legally hunted in the Tejada and Almijara Game Reserve, in southern Spain (36°N latitude, Province of Malaga, Spain) during the rutting season (November/December 2013/2014). Epididymal spermatozoa were collected by the retrograde flushing method, using 1 mL of Tris-citric acid-glucose medium (TCG) at ambient temperature (11-13°C in the field laboratory). Sperm from left epididymis were frozen with TCG-6% egg yolk and 5% glycerol, and sperm from right epididymis were vitrified with TCG-6% egg yolk with 100 mM sucrose. There weren't differences between treatments (frozen-thawed vs vitrified-warmed sperm) for the percentage of motile sperm, percentage of sperm with membrane integrity determined by the hypo-osmotic swelling test, and percentage of sperm with morphological abnormalities (%). However there were significant differences for quality (score 0-4) of motility (2.4 ± 0.2 and 1.4 ± 0.2), percentage of progressive motility (22.7 ± 4.3 and $7.0 \pm 1.6\%$), percentage of intact acrosome (73.8 ± 4.0 and $55.9 \pm 2.5\%$), percentage of viable sperm according to the nigrosin-eosin staining (45.5 ± 4.1 and $29.2 \pm 4.1\%$), percentage of dead sperm with damaged acrosome (5.5 ± 1.0 and $17.3 \pm 2.3\%$) and percentage of live sperm with intact acrosome (45.1 ± 5.5 and 26.5 ± 4.6) respectively. Although better results were found using the conventional freezing method, given the simplicity of sperm vitrification its use under certain field conditions can be recommended for this type of species. Improvement of the technique might, however, provide better post-vitrification outcomes; new vitrifying solutions and additives should be assessed in future work.



A025E Physiology of Reproduction in Male and Semen Technology

β -defensin 126 and sperm function in cattle

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Keywords: β -defensin 126, cattle, cauda epididymis, sperm migration.

β -defensins are antimicrobial peptides also thought to have a role in sperm function. In cattle, β -defensin 126 (BD126) has been only detected in the male reproductive tract, with preferentially in the epididymis (Narciandi et al., Immunogenetics 63, 641–651, 2011). The macaque ortholog has been shown to enhance the ability of sperm to migrate through cervical mucus (Tollner et al., Hum. Reprod. 23, 2523–2534, 2008). A mutation in the BD126 gene has been linked to subfertility in men, only explained by reduced ability to penetrate through mucus *in vitro* (Tollner et al., Sci. Transl. Med. 3, 92ra6, 2011). The aim of this study was to examine the role of bovine BD126 in sperm function. Western blot (WB) analysis with a BD126 specific monoclonal antibody demonstrated significant BD126 on bovine sperm which previously published methods for macaque sperm failed to remove. WB analysis also revealed that while BD126 is present on sperm and in seminal plasma from intact bulls, it is undetectable in the ejaculate of vasectomised animals, indicating that it does not originate in the accessory glands. Further analysis demonstrated that the peptide is uniquely present in the cauda epididymis and is absent from sperm recovered from other epididymal regions, thus providing a model to study its function. Confocal analysis revealed immunofluorescent labelling of BD126 specific to the tail and acrosomal region in cauda sperm only, suggesting a role in sperm motility. We therefore hypothesized that addition of cauda fluid to corpus sperm would improve motility and ability to penetrate cervical mucus *in vitro*, and that this may be due to the activity of BD126. Testes were collected from adult bulls at an abattoir and sperm from the corpus and cauda epididymis, as well as cauda epididymal fluid (CEF), were recovered. Corpus sperm were incubated for 1 h with CEF in the absence or presence of BD126 antibody (Ab); untreated corpus and cauda sperm were used as controls. A higher number of cauda than corpus sperm migrated through cervical mucus ($P < 0.001$) and addition of CEF increased the number of corpus sperm migrating through this matrix ($P < 0.05$). The presence of the BD126 Ab failed to abrogate this effect. Analysis of motility using a computer assisted sperm analysis system indicated higher total and progressive motility in caudal sperm when compared with sperm from the corpus ($P < 0.001$); again, addition of CEF increased progressive motility ($P < 0.05$). In conclusion, we have characterised the expression of bovine BD126 as a protein in the cauda epididymis. Incubation of sperm from the corpus epididymis (which lack BD126) with CEF from the cauda (which contains BD126) resulted in enhanced sperm migration through cervical mucus, and higher motility. Further work will clarify the role of BBD126 and related β -defensins in mediating bovine sperm function.

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A026E Physiology of Reproduction in Male and Semen Technology

Expression of β - nerve growth factor in rabbit male tract and seminal plasma

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Keywords: β -NGF, male reproductive tract, seminal plasma, rabbit

Nerve growth factor (NGF) has been recently identified as an ovulation inductor factor (OIF) in the seminal plasma (SP) (Ratto et al. PNAS 2012; 109:15042-7). The presence of OIF in rabbit has been suggested but this protein has not yet been identified. Our aim was to study the mRNA expression in the rabbit male reproductive tract and to identify the protein β -NGF in the SP. Total RNA was extracted from prostate, testicles and seminal glands of 3 male rabbits (TRIzol® Plus RNA Purification Kit, Life Technologies) to subsequently isolate mRNA (FastTrack® MAG mRNA Isolation Kit, Ambion, Life Technologies,) for retrotranscription to generate cDNA. Specific primers were designed on the mRNA sequence deposited in GenBank (XM_008264614.1) to target a highly conserved region of NGF among species (5'-AGCCCACTGGACTAAACTGCA-3'; 5'-TCGCACACCGAGAACTCTCC-3'; product size: 305 nucleotides). PCR was performed on cDNA to obtain the expected 300 pb fragment that was sequenced confirming the presence of NGF-mRNA in seminal plasma, testicle and prostate. To determine the expression of mature NGF protein in SP, an aliquot was prepared from collected semen, centrifuged at 3000xg for 30 min at 4°C and stored at -20°C. For Western blot (WB) analysis, samples were loaded in 12% SDS-PAGE and electrotransferred onto nitrocellulose membranes. The membranes were probed with mouse β -NGF antibody (Promega) using donkey anti-mouse as secondary antibody (Li.Cor Biotechnology). Blots were scanned in an Odyssey Infrared imaging system. In addition, NGF was purified by offgel technique with the 3100 OFFGEL Kit pH 3-10 (Agilent Technologies Inc) and the recovered fraction recognized with the mouse β -NGF antibody was used for mass spectrometry analysis (MS) (4800 Plus Proteomics Analyzer Applied Biosystems,). MS was operated in positive reflector mode with an accelerating voltage of 20,000 V. For protein identification NCBIInr was used. Database without taxonomy restriction and a home-made database with the sequence of NGF (gi|655847230) downloaded from NCBIInr was searched using MASCOT v 2.3. The probability scores of NGF sequences from several species were greater than the score fixed by MASCOT as significant with a p-value < 0.05. Our results show that expression of NGF-mRNA were clearly identified in the rabbit male tract organs above described and the corresponding mature protein band with a mass of ~60 kDa was also identified by WB whereas a ~13 kDa band was detected in the basic fraction (pH=8.24-8.83) obtained when offgel electrophoresis was performed. Furthermore, protein identification by mass spectrometry revealed the existence of NGF in the SP. In conclusion, mRNA and protein NGF are present in rabbit male reproductive tissue and SP respectively, providing the basis to undertake further functional analysis for its potential role in rabbit reproduction.

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A027E Physiology of Reproduction in Male and Semen Technology

Assessment of bull semen quality loaded in new SensiTemp straws using semen and IVP technologies

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Keywords: bull semen, fertilization, IVP, SensiTemp straws.

SensiTemp, a new IMV bull straw concept, presents the advantage of color changing while the straw is thawed. The color of frozen straws is blue and straws start to become white when the temperature reaches 33°C, with a complete change of color at 37°C. The objective of this study is to assess quality after thawing of semen frozen in SensiTemp, *in vitro*, using Computer Assisted Semen Analysis (CASA), Flow Cytometry (FC) and *In Vitro* Fertilization (IVF). The ejaculates of two bulls, selected during preliminary experiments on high *in vitro* fertility, were harvested at CIA L'Aigle, France and split ejaculates were frozen in experimental (SensiTemp) and conventional (Control) straws. In experiment 1 after thawing semen from the two type of straws (5 *pooled straws each*; 2 *replicates*), motility was assessed using the IVOS CASA system (Hamilton Thorne Inc., Beverly, MA, USA) and membrane integrity was evaluated through FC with Cytosoft software (Millipore-Guava Technologies Inc., Hayward, CA). In experiment 2, IVF was used to evaluate the non toxicity of SensiTemp and control straws. Cumulus-oocyte complexes (COC; $n=1178$; 4 *replicates*) collected from slaughterhouse ovaries were matured in IVM medium (TCM-199 with bicarbonate, Sigma-Aldrich, Saint Quentin Fallavier, France; 10µg/ml FSH-LH, Reprobiol, Liège, Belgium and 10% FCS, Thermo Fisher, Illkirch, France) for 22 h. After fertilization, presumptive zygotes of each group (SensiTemp and control for each bull) were cultured in synthetic oviduct fluid medium (SOF, Minitube, Tiefenbach, Germany) with 1% ECS and 0.6% BSA (Sigma-Aldrich, France) up to 8 days. All cultures were conducted at 38.5C in 5%CO₂, 5%O₂. The cleavage and blastocysts rates were evaluated on Day 3 and 7, respectively for each group. Embryo quality was recorded on day 7 according to the IETS evaluation. Data from each bull were analyzed separately using the Chi square test ($P<0.05$). In experiment 1, neither sperm motility from bull 1 (61.2 and 60.5%) and bull 2 (66.2 and 66.5%) nor membrane integrity from bull 1 (58.6 and 52.2%) and bull 2 (61.0 and 61.9%) were different between SensiTemp and Control, respectively. Results from experiment 2 showed no difference ($P>0.05$) in cleavage rate between SensiTemp and Control for the two bulls: 92.1 and 91.7% for bull 1 and 94.2 and 94.6% for bull 2 respectively. The blastocysts rate on day 7 did not differ ($P>0.05$) among groups (47.5, 47.1 and 51.3, 50.4% for SensiTemp and Control bull 1 and bull 2, respectively) nor the quality of embryos retrieved in the different groups: 25.4, 23.3 and 30.8, 29.6% in grade 1 embryo for SensiTemp and Control bull 1 and bull 2, respectively. Those results demonstrate, *in vitro*, that the new SensiTemp straws were non toxic and did not affect the semen quality after thawing nor did the SensiTemp straws affect the ability of sperm cells to fertilize oocytes and produce 8 days old embryos.



A028E Physiology of Reproduction in Male and Semen Technology

Hyaluronic acid-binding ability of spermatozoa and its role for selection of vacuole free human spermatozoa in human reproduction

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Keywords: ART outcomes, DNA integrity, sperm head vacuoles, MSOME, sperm selection, hyaluronic acid binding.

The type of spermatozoa selected in ART (assisted reproductive technology) influences the outcome in regard to embryo development, pregnancy, miscarriage and malformation. Sperm head nuclear abnormalities were identified earlier as vacuoles by motile-sperm organelle-morphology examination (MSOME). Blastocyst development and the pregnancy rates are negatively influenced if vacuoles containing sperm are used for ICSI. Thus, it is of importance to reliably select vacuole-free spermatozoa in assisted reproduction. In a prospective, observer blinded study. Hyaluronic acid (HA) bound, standard morphological (SM) selected (200x) and unselected sperm were collected by different examiners. The evaluation of vacuoles by Nomarski differential interference contrast (DIC; 600x up to 7.200x) was performed observer blinded for all samples. Eleven human semen samples were prepared by a 80% density gradient. From each sample a minimum of 20 sperm per method (HA and SM selection) were collected in separate PVP droplets. Additionally, 20 unselected spermatozoa were collected from each sample designated as control. The number of vacuoles in each sperm head was determined by means of DIC. One way analysis of variance was performed (Tukey-Test; Sigma Stat Version 3.5, DUNDAS Software LTD.). Significantly more sperm without vacuoles were found in HA selected ($p < 0.001$) and SM selected ($p < 0.001$) than in unselected samples. The number of sperm with one or two vacuoles ($p < 0.01$) and more than two vacuoles ($p < 0.001$) was significantly higher in the unselected group. Furthermore, in HA selected sperm the appearance of two vacuoles was significant lower than in SM selected sperm ($p < 0.05$). Both selection methods provide spermatozoa containing less vacuoles than in the unselected samples, especially in the group with more than two vacuoles. This shows that HA selection is a good method to select spermatozoa in regard to the appearance of vacuoles. This is of significance since the HA selected spermatozoa are more mature, with less cytoplasmatic retention and higher DNA integrity than unselected sperm cells. Thus HA selection may be an effective method to identify spermatozoa with a higher potential in reproduction in order to improve safety and results in ART procedures.



A029E Physiology of Reproduction in Male and Semen Technology

Seasonal variation of testicular functionality in alpaca (*Vicugna pacos*) raised in Italy

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Keywords: alpaca, scrotal edema, testicular measures, ultrasonography.

Thermoregulatory functions of testicles are very important for sperm viability in terms of spermatogenesis and maturation phases. Unique characteristics are present in South American camelids related to the position, body mass/testicle volume ratio and anatomical features (epididymis orientation). Among different parameters to evaluate male there are testicular dimension measures (width, length and thickness). It has been observed different times the increase of testicular volume during heat season in animals with pendulous testicles because of circulatory impairment. Aim of this study was to monitor physical and physics parameters besides the semen quality evaluation during two different seasons (summer, winter) in alpacas. Eight adult males are evaluated considering classical (testicular measures – caliper measurements) and innovative parameters as ultrasonography of the testicles. Semen collections were performed with a teaser and ejaculates obtained were destined to the quality assessment (volume, colour, viscosity, motility and concentration) and biochemical evaluation of the seminal plasma (energetic, protein and enzymatic profile – Hitachi 912 biochemical auto-analyzer). Data were analysed for ONE-WAY ANOVA considering the season as variable independent and the parameters evaluated as dependent variables using the statistical software SIGMASTAT 2.05. There was a significant difference among seasons with a general decrease of the semen quality during the hot season. The lower levels of volume, concentration, seminal plasma (SP) glucose, SP cholesterol, SP triglyceride, SP Phosphates and the higher levels of SP Gamma Glutamyl Transferase, SP Alkaline Phosphatase, SP Magnesium clearly indicate a detrimental effect of high environmental temperature because the effect on testicular thermoregulatory capability. Negative correlation between Testicular Measures and semen quality parameters was significant ($r: -0.64 - -0.45$). At the ultrasound evaluation was characterized the reason of increased testicular mass during the hot season considering the evidence of scrotal edema. The scrotal edema derived by a defect of local circulatory mechanism. Testicular functionality may be influenced by the high environmental temperature and specifically in alpaca were the position of the gonads imposes a fine regulatory pattern. Hot season causes a testicular circulatory defect with a scrotal edema as results and a decrease of semen quality.

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A030E Physiology of Reproduction in Male and Semen Technology

Characterization of accessory glands ultrasonography in rams of endangered venetian sheep breeds

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Keywords: accessory glands, endangered Venetian Sheep Breeds, breeding soundness evaluation, ultrasonography.

Evaluation of male needs standardized protocol for the male's classification. Breeding soundness evaluation (BSE) is a practice that is widely used, mainly in the bull, to evaluate the male starting from physical and reproductive parameters. BSE protocols for rams are already published around the world but complete evaluation is not yet raised for all the breeds with specific characteristics. The male can be classified as Satisfactory, Questionable and Unsatisfactory. When is not possible to evaluate it properly, the classification can be deferred. Among the innovative methods to perform the evaluation, there is the ultrasound exam of the reproductive organs. Testicles, epididymis, vascular cone and accessory glands ultrasound may increase the accuracy of the evaluation. In this study, an established sample of rams belonging to Veneto Agricoltura Center in Villiago (BL), Italy, has been evaluated with classical and innovative monitoring system. On these animals the entire BSE procedure was carried out. Moreover, the ultrasound evaluation (MyLabVetTM One, ESAOTE S.p.A., Genova, 10 MHz probe frequency) of testicles and vesicular glands has been performed for the first time in these breeds (18 adult rams: N=5 Brogna, N=5 Lamon, N=4 Foza, N=4 Alpagota). After the physical and physics exams all the males involved in the evaluation were collected using electro-ejaculator (Ruakura Ram Probe Plastic Products, Hamilton, New Zeland); the trans-rectal probe was inserted after a mucosal anesthesia (5 ml of Lidocaine 2 %) performed during the deferent ampullas massage. Procedure of semen quality evaluation considering general ejaculate parameters (color, volume, concentration) and specific microscopic observation about viability fresh-post thawed with differential staining (Eosin/Nigrosin, Spermac and Farrelly staining), kinetic CASA parameters (Ivos II, Hamilton Thorne, Germany). Data analysis (Pearson correlation indices) revealed important correlations among scrotal circumference, serum testosterone and semen kinetic parameters. Furthermore, increasing the testicular parenchyma echogenicity, the semen volume used to lower. Testicular and vesicular glands ultrasound exam give us important information about seminal plasma quantity. Particularly vesicular glands echogenicity has shown high relationship with quantity of seminal plasma and therefore low sperm concentration. Physics equipment as ultrasonography may optimize collection procedure performed with electro-ejaculator. Body mass and vesicular glands dimension can influence the induction success and the semen freezeability.

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A031E Physiology of Reproduction in Male and Semen Technology

The joint treatment of sperm by prolactine and GTP have determined the increase of the number acrosome-reacted spermatozoa in bulls

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Keywords: acrosoma-reacted spermatozoa, bulls, prolactin.

There are contradictory opinions concerning involvement of prolactin (PRL) in the process of sperm capacitation and acrosome reaction (Vigil P. et al., 2011 Biol Res, 44:151-159). It was shown that PRL stimulates release of Ca^{2+} from IP₃-sensitive stores, and GTP stimulates release of this ion from IP₃-insensitive stores (Denisenko V. et al., 2015 Tsitologiya, 3:1-8). GTP forms a connection between IP₃-sensitive and IP₃-insensitive intracellular stores and promotes transition of Ca^{2+} between these stores (Mullaney J. et al., 1987 J. Biol. Chem. 262: 13865—13872). The aim of the present study was to examine the mobilization of Ca^{2+} from intracellular stores after the treatment spermatozoa by PRL and GTP and to evaluate the status of spermatozoa after these treatments. Intensity of fluorescence of membrane-bound Ca^{2+} was determined with a fluorescence spectrophotometer Hitachi MPF-4 (excitation: 380-400nm, emission: 530 nm) using 40 μ M chlortetracycline (CTC) - (Denisenko V. et al., Tsitologiya 3:1-8, 2015). Intensity of fluorescence of membrane-bound Ca^{2+} was determined in Sp-TALP medium where the concentration of cells was adjusted to 1, 5 X 10⁶ sperm/mL. The CTC assay was used to determine the functional status of spermatozoa (Ded L. et al., 2010 Reprod Biol Endocrinol, 8-87). Samples were examined with fluorescence microscope Zeiss Axo Imager M1. Ejaculates from three fertile bulls were used, and five replicates were performed for each experiment. In each sample, 200 cells were evaluated. Sperm were evaluated according to 1 of 3 CTC staining patterns: fluorescence over the entire head (precapacitated cells), fluorescence-free band in the postacrosomal region (capacitated cells) and low fluorescence over the entire head except for a thin bright fluorescent band along the equatorial segment (acrosome-reacted cells). All reagents that were used in this study were produced by Sigma-Aldrich (Moscow, Russia). Data were analyzed by Student's t-test. Treatment spermatozoa by PRL (10 ng/ml) or GTP (10 μ mol) resulted in release of Ca^{2+} from intracellular stores (0.70 \pm 0.019 and 0.69 \pm 0.017 vs 0.85 \pm 0.016; P<0.001). There was additional release of Ca^{2+} with the combined effect of PRL and GTP (0.62 \pm 0.011 vs 0.70 \pm 0.019 and 0.69 \pm 0.017; P<0.001). There was no additional release of Ca^{2+} after the joint action by the pair of these reagents in the presence of protein kinase C inhibitor (Ro 31-8220, 10ng/ml). The average percentages of capacitated spermatozoa did not change after treatment by PRL, GTP or both these reagents. The percentage of cells that underwent acrosome reaction have increased after treatment by PRL and GTP jointly (46% vs 62%, P<0.01); there was no such effect at preliminary treatment of sperm by Ro 31-8220 (10 ng/ml). Thus, Ca^{2+} transition between intracellular stores in bull spermatozoa after the treatment with PRL and GTP jointly is leading to increasing in the percentage of acrosome-reacted spermatozoa.