(65°C/6 s) by CASA and flow cytometry (PI/PNA–FITC and SYBR–14/PI). Results are represented as mean ± SEM. Values of $p < 0.05$ were considered statistically significant. Total motility (%) showed higher values for EY20, LDL5 and LDL10 ($p < 0.05$). Progressive motility (%), curvilinear velocity (μm/s) and straightness velocity (μm/s) were higher for EY20, but linearity index (%) was higher both for EY20 and LDL15. Contrasting with these results, flow cytometry showed that LDL might provide better plasma membrane protection than egg yolk. Viable spermatozoa (SYBR-14 ± PI−) were higher for both LDL10 and LDL15 (EY20: 39.2 ± 6.3; LDL5: 33.1 ± 4.3, LDL10: 48.4 ± 3.3 and LDL 15: 59.4 ± 4.7) whereas the proportion of damaged acrosomes (PNA− FITC+) showed no differences. The differences among motility and flow cytometry results may be caused by the presence of coarse particles in the LDL extenders, which might have increased the proportion of events identified as non-motile spermatozoa by the CASA. The use of LDL at both 10% and 15% might be a substitute of egg yolk, but it would be necessary to improve the purification process of LDL in order to prevent the presence of large particles in the extender. Supported partly by CICYT (CGL2010–19213/BOS), Cantur SA and Ramón y Cajal program (RYC–2008–02560, MICINN, Spain).

Key Words: LDL, brown bear, cryopreservation, sperm

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Conservation of genetic potentials of Eastern sarus crane (*Grus antigone sharpii*): effects of season and preservation techniques on semen quality

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Eastern sarus crane is classified as vulnerable on the IUCN Red List. While only a few thousands of Eastern sarus cranes have been estimated worldwide, they have become extinct from natural habitats of several countries including Thailand. This study was conducted to examine the influence of season on semen traits and also to investigate the effects cold storage and cryopreservation on semen quality of Eastern sarus crane. During April 2010 to September 2011, semen from 6 captive cranes was manually collected every 2–3 weeks at Nakhon Ratchasima zoo, Thailand (14°59′N, 102°12′E). The semen characteristics were examined. In experiment 1, the semen was diluted with crane semen extender and cooled in a refrigerator to 4°C. The semen was then examined for motility and viability at 24, 48 and 72 h after cold storage. In experiment 2, the semen was cryopreserved using different cryoprotectant (dimethyl sulphoxide, DMSO) equilibration techniques (1-step vs. 2-step). The frozen semen was then thawed by two techniques (37°C vs. in-air thawing) prior to examine the sperm motility and viability. The sperm concentration ranged from 0.03 ± 0.08 to 8.7 ± 10.8 ($\times 10^6$ per ejaculation) with a large variation of semen volume (1.8 ± 21–109.5 ± 68.4 μl). The increase in sperm quantity and quality (viability and motility) from June to August (rainy season) compared with April (summer) appeared to coincide with an increase of rainfall, indicating the effects of season on semen production. Cold storage significantly decreased the sperm quality over the times of cold storage. The viability and motility of semen exposed to 48 h of cold storage were significantly lower than fresh semen (58.5 ± 9.5% and 50.0 ± 0% vs. 74.0 ± 12.5% and 66.1 ± 10.2%, respectively). DMSO equilibrating techniques (1- vs. 2-step) did not significantly differ in terms of sperm motility and viability, when the semen was thawed by a particular technique. However, the 2-step DMSO equilibration and in-air thawing significantly increased the percentage of sperm motility (41.5 ± 11.6%) compared with 37°C thawing (33.0 ± 8.7%), but the viability was not affected. It is concluded that semen characteristics of Eastern Sarus crane is influenced by seasonality. We have described for the first time that cold storage is an efficient preservation technique for maintaining the semen quality of Eastern Sarus crane for at least 48 h, while cryopreservation is more detrimental to sperm quality. Fertility test of cooled/frozen-thawed semen by means of artificial insemination remained to be performed.

Key Words: Eastern sarus crane, season, semen, cryopreservation

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Effects of alternative cryoprotectants, diluents, straw size and cholesterol addition on cryopreserved rooster sperm

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Rooster sperm do not cryopreserve well. This is due in part to osmotic changes that sperm undergo during addition and removal of cryoprotectant (CPA), as well as membrane damage during the membrane phase transition from fluid at 37°C to gel at low temperatures. When a CPA is removed from sperm, it induces a transient osmotic gradient across the plasma membrane as CPA permeability is lower than that of water. An alternative CPA, methylacetamide (MA), with a lower molecular weight than glycerol (GLY) permeates the plasma membrane more quickly than GLY, potentially decreasing osmotic damage. To decrease membrane damage induced by the membrane phase transition, cholesterol can be added to plasma membranes, increasing membrane fluidity at lower temperatures. Rooster sperm were collected from several birds, pooled and diluted to 1 billion cells/ml in either a trehalose-based diluent (TD) or glutamate-based diluent (GD). In experiment 1, sperm were diluted 1:1 at 5°C with either 18% GLY or 18% MA resulting in a 9% CPA final concentration. Sperm were packaged in 0.25 or 0.5 ml straws and frozen in LN vapor. Motility analyses were conducted using CASA for sperm thawed in a 5°C water bath and diluted 1:10 in GD or TD containing 10% BSA. Data for all experiments were analyzed by ANOVA and means separated using Student–Newman–Keuls multiple comparison test. Higher motility rates were seen for treatments packaged in 0.5 ml straws ($p < 0.05$). In addition, for sperm frozen in 0.5 ml straws, cells frozen in TD exhibited higher motility (>60%) than sperm frozen in GD (46–53%). In experiment 2, cholesterol was added to sperm using cholesterol-loaded cyclodextrins (CLCs) at 0.5, 1, 2 and 3 mg/ml after initial sperm dilution and incubating at 5°C for 30 min. Sperm were then diluted 1:1 with either 18% GLY or 18% MA in either GD or TD, packaged in 0.25 ml straws and frozen. Addition of CLCs did not improve sperm post-thaw motility rates ($p > 0.05$). To determine if MA exposure is detrimental to sperm, in experiment 3, sperm were exposed to 9% MA at 5°C in TD for 0, 2.5, 5 and 10 min prior to freezing. MA exposure time prior to cryopreservation did not affect post-thaw sperm motility rates ($p > 0.05$). In experiment 4, sperm were left with 9% MA for 0, 5, 10, 15, 20, 30, 45 and 60 min after thawing, before analysis. Exposure to MA for up to 1 h post-thaw did not affect sperm motility rate ($p > 0.05$). In conclusion, altering sperm membrane composition by adding CLCs did not improve post-thaw motility of rooster sperm. Sperm frozen in TD with 9% MA or 9% GLY in 0.5 ml straws protected the cells from cryodamage most effectively, and exposure to MA prior to or after freezing did not affect sperm motility. Future experiments will determine the fertilizing capacity of sperm frozen using MA as the cryoprotectant.

Key Words: Poultry, sperm, cryoprotectants, cryopreservation

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The effect of omega-3 fatty acids addition in sperm extenders on the quality of frozen bovine semen

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The role of omega-3 polyunsaturated fatty acids (n-3 PUFAs) on sperm resistance to cooling and storage is controversial. Although previous studies demonstrated that the addition of omega-3 fatty acids to the diet of animals (e.g. pig and horse) can influence sperm membrane fluidity and integrity as well as male fertilizing capacity,

there is not enough data about their effects on semen quality *in vitro*, especially on bovine semen. So, the present experiment was designed to determine the protective effects of various levels of purified n-3 PUFAs on bovine semen quality *in vitro* in response to cryopreservation. Polyethylene glycol (PEG), as a more suitable solvent has to be added to introduce n-3 PUFAs to semen extenders. Media were finally sonicated. In treatment A, PEG was added alone to the samples and in treatments B, C and D three different concentrations of n-3 PUFAs (1%, 2.5% and 5%) in combination with PEG were added to the semen extender (Tris-citrate buffer medium containing egg yolk and glycerol). Five proven bulls were randomly selected; ejaculates were collected by artificial vagina and semen characteristics were recorded. Only ejaculates with normal characteristics (volume > 5 ml; concentration > 1.2 billion/ml; motility > 75%; normal morphology > 85% and viability > 85%) were chosen and diluted semen (40 millions/ml) was loaded into 0.5-ml straws, sealed and frozen. Motility, viability and morphology were investigated in frozen-thawed sperm after 1 month. Motility and other dynamic parameters of sperm were analyzed by computer aided sperm analysis (CASA). The results were evaluated by repeated measure ANOVA using SPSS and $p < 0.05$ was considered significant. Motility were 37.9%, 12.8%, 13.2%, 13.8% and 10.4%; viability were 54.8%, 25.7%, 27.6%, 26% and 19.2%; and normal morphology were 88%, 72.7%, 80.6%, 75.4% and 83.6% for control, treatments A, B, C and D, respectively. Our results showed that PEG has some detrimental effects on sperm motility and viability, whereas the addition of n-3 PUFAs to semen extenders could not attenuate the detrimental effects of PEG and did not significantly ($p < 0.05$) improve bovine sperm quality *in vitro*. It seems that n-3 PUFAs cannot be effectively introduced to conservation media as well as sperm membrane to modify sperm characteristics. Nonetheless, n-3 PUFAs might rather be supplemented to the diet of bulls in order to modify the fatty acid compositions of sperm and perform their preventive properties.

Key Words: Bovine, sperm quality, omega-3 fatty acids, freezing

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Field fertility results of sublethal stress treated frozen-thawed boar semen using commercial cervical insemination without ovulation induction

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Former studies reported enhanced cryosurvival of boar spermatozoa if sperm was treated with sublethal hydrostatic pressure (HP) stress before freezing (Pribenszky et al., 2007; Huang et al., 2009; Horvath et al., 2011). The present experiment aims to investigate the effect of HP treatment of fresh semen before cryopreservation on pregnancy rate, litter size, and litter characteristics. Semen was collected from four boars with 'good' freezability. Sperm rich fraction was extended with commercial extender at 35°C (BT). Diluted semen was cooled to room temperature (RT) then centrifuged at 2400 × *g* for 3 min. Pellets were re-extended with Ext II. (lactose + egg yolk), then split to treatment and control groups individually. Treatment groups were filled individually into plastic syringes closed by a plastic cap and were treated with 40 MPa HP for 80 min at RT in a programmable hydrostatic pressure machine (HHP1400, Cryo-Innovation Ltd. Hungary). After treatment, Ext III was added (Ext II. + glycerol and Equex Paste), giving a final concentration of 6% glycerol and 1 × 109 spermatozoa/ml. Semen was then loaded to 0.5 ml straws, sealed and were placed into a cooling cabinet at 15°C for 1 h. Samples were further cooled to 5°C for 2 h. Straws were then placed 4 cm above liquid nitrogen for 20 min followed by plunging and storing. Control semen was frozen without HP treatment.

Straws were thawed at 37°C for 30 s, extended with Ext. I up to 80 ml. Motility was checked by CASA (Minitube, Germany). 103 sows that came to heat at day 5 after weaning were selected and randomly assigned to the HP or control groups. Insemination was done through 10 consecutive weeks, on day 5 after weaning at 6 am and 4 pm using commercial AI catheter, without any ovulation induction, with 6 × 109 frozen-thawed semen. Pregnancy was checked by ultrasound on day 25 after AI. Piglet count and weight measurements were done after farrowing, after weaning at day 28 days and at day 42. Results were analysed by genralised mixed model. Results: 81.6% of HP and 60.8% of control sows became pregnant (OR = 2.9, $p = 0.024$). Progressive and total motility were higher in HP samples compared to controls ($p < 0.001$), however these parameters did not differ significantly between pregnant and non-pregnant sows. HP sows gave birth to significantly higher ($p = 0.008$) number of pigs compared to control sows (HP: 10.8 ± 4.5 vs. control: 8.0 ± 3.8). The ratio of stillbirth pigs did not differ between groups (HP: 13.7 ± 18.1% vs. control: 9.3 ± 10.6%). The daily gain of the HP piglets was higher compared to the control group in the fattening stage (290 g/day vs. 275 g/day), where feed was supplied *ad libitum*. Conclusion: HP treated frozen-thawed semen inseminated in the routine workflow of a commercial swine farm with commercial catheter and routine procedures resulted in pregnancy rates and litter size comparable to fresh semen insemination. Study was supported by KMOP-1.1.1.-08/1-2008-0065

Key Words: Boar, semen, cryopreservation, fertility

07. Early embryonic development:

0700

Effect of bovine oviductal epithelial cell co-culture on early cleavage kinetics of bovine IVP embryos

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The use of bovine oviduct epithelial cells (BOEC) as a co-culture system for cattle embryos has been widely used to study embryo maternal interactions and to improve embryo quality during IVP. However, the exact mechanisms of BOEC action have not been fully elucidated. We hypothesize that somatic cells could finely tune the early stages of embryo development, allowing embryos to go faster through the first cleavages, embryonic genome activation and finally reaching the blastocyst stage earlier. Therefore, the purpose of this study was to assess the protein secretion, gene expression and morphology of bovine oviductal epithelial cells according to the time of culture, and to evaluate, for the first time, the effect of BOEC co-culture on early cleavage of bovine embryos. Confluent monolayers of bovine oviductal epithelial cells (BOEC) were derived from slaughterhouse oviducts. Immature COC were aspirated from slaughterhouse ovaries. Zygotes were produced by in-vitro maturation and fertilization, and cultured in SOF medium supplemented with 10% FCS in the presence of BOEC or not. Four development groups were compared: (C) control, medium alone, (BE) BOEC during 4 days, then medium alone, (BL) medium alone for 4 days, then co-culture for the last 4 days, (B) BOEC co-culture over 8 days. Blastocyst rate was evaluated at Day 8. Cell numbers were evaluated at 96, 115, 120 and 139 h post fertilization, using Hoechst 33 342 fluorescent staining for embryos cultured in groups C and B. Oviduct protein secretion and gene expression (OSP, OVGP, C3 and GPX4) were evaluated at confluence and at the end of the culture period. Morphology of BOEC (tubulin, actin and cx43) was assessed by confocal microscopy.

Cleavage rate at 48 hpi was not significantly different between groups. Co-culture improved blastocyst rate compared to control group (C: 13%, B: 25%, $p < 0.05$). The blastocyst rate was higher in group BE compared to BL (36% vs. 25%, $p < 0.05$). The mean cell number at different times of culture did not differ significantly between treatment groups. However, the rate of embryos with 20 or more cells was significantly increased in B group as compared to C at 115 (C: 14.1%, B: 21.8%, $p < 0.05$) and 139 hpi (C: 25.9%, B: 42.2%, $p < 0.0001$). Furthermore, it was observed that co-cultured zygotes reached the 20–25 cell stage embryos (maximum at 115 hpi) sooner than the control group (maximum at 120 hpi). BOEC protein secretion, gene expression and morphological assessment suggested a functional maintenance of BOEC differentiation over the culture time. In conclusion, BOEC co-culture improved in-vitro embryo development. This BOEC effect seems more critical during early stages. Furthermore, BOEC co-culture was accelerating the kinetics of the first cleavages sequence, allowing embryos to reach faster the 20-cell stage, compared to controls. All together, these data suggest a possible role of BOEC in the regulation