A067E Folliculogenesis, Oogenesis and Ovulation

# Osmotic challenge of bovine early pre-antral follicles with different cryoprotectant agents

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**Keywords**: bovine preantral follicle, cryoprotectant agent, DMSO, EG, follicle permeability, osmotic challenge.

Significant advances in cancer diagnosis and treatments have stimulated interest in fertility preservation strategies as chemical or ionizing radiotherapy often threatens future reproduction. Ovarian tissue cryopreservation currently is the only option for preserving the reproduction potential of pre-pubertal girls and women whom cannot undergo hormonal stimulation, ovarian tissue cryopreservation currently is the only option. However, there is a huge concern regarding the possible presence of malignant cells in the retrieved ovarian tissue, which could lead to cancer reintroduction after reimplantation of the frozen-thawed tissue strip. Cryopreservation of isolated early preantral follicles (PAFs) (and subsequent *in vitro* culture (IVC), maturation (IVM) and fertilisation (IVF)) might therefore represent an interesting alternative. Existing protocols are based on protocols for freezing embryos and oocytes . However, in order to improve follicular survival after cryopreservation, it is essential to develop a protocol for follicles specifically. Indeed, follicles are quite different from both embryos and oocytes, if only because they are composed of two different cell types (namely the oocyte and the surrounding (pre-)granulosa cells). In order to provide a biophysical base for choosing optimal cryoprotectant agents (CPAs) that avoid severe volume changes and formation of intracellular ice crystals, in this experiment, two-day-old isolated bovine PAFs were osmotically challenged by exposing them to different concentrations of cryoprotectant agents (CPAs). Briefly, isolated bovine early PAFs were exposed to either ethylene glycol (EG) or dimethyl sulfoxide (DMSO) in different final concentrations: 1 M, 2 M, 3 M, 4 M and 5 M at room temperature, and photographed at different time points (every half minute between 0 and 5 minutes) after the onset of exposure to calculate their volume over time (5 - 10 follicles per CPA and per concentration). Although there was a high variability in the individual response of the follicles to this CPA challenge, all follicles showed a typical ‘shrink/swell’ curve. Analysis with two-way ANOVA showed no interaction between the type of CPA and the respective concentrations. This means that volume differences in time between the minimum and maximum for both EG and DMSO were uniform across concentrations. Across all concentrations, time until the post stimulus maximum (i.e. the maximum volume to which follicles re-expand after shrinkage) appeared significantly longer in the EG group ($P$ = 0.04), indicating that bovine early PAFs are less permeable to EG than DMSO. To our knowledge, this is the first time that isolated bovine early PAFs were osmotically challenged by exposing them to different concentrations of penetrating CPAs. This has provided us with some basic insights in follicular permeability to CPAs. These insights are a first step in the design of cryopreservation protocols for isolated early PAFs specifically.

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# Developmental competence of porcine oocytes that have finished growth phase from follicles of different diameter

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**Keywords**: Brilliant cresyl blue, diameter, follicular development, pig oocytes.

Numerous factors determined developmental competence of the oocytes. Brilliant cresyl blue (BCB) staining has been used for selection of oocytes from several mammalian species, including pigs (Ericsson S. et al, 1993 Theriogenology, 39(1): p.214). The aim of the present study was to evaluate the developmental competence of porcine oocytes that have finished growth phase ($BCB^+$) depending on diameter (d) of follicles (d < 3 mm, 3 - 6 mm, >6 mm) and to detect expression of estrogen receptor (ER) in cumulus cells of $BCB^+$ and $BCB^-$ oocytes. Before IVM compact cumulus oocyte complexes (COC) were incubated in BCB solution for 60 minutes. Treated oocytes were divided into $BCB^-$ (colorless cytoplasm) and $BCB^+$ (colored cytoplasm). Only $BCB^+$ oocytes were used in the experiments. The medium used for oocyte maturation was NCSU 23 supplemented with 10% follicle fluid (FF), 0.1 mg/ml cysteine,10 IU/ml eCG and 10 IU/ml hCG. FF was collected from follicles with 3 - 6 mm in diameter, COC cultured in maturation medium with pieces of wall (600-900 μm in length) from non atretic healthy follicles (d 3-6 mm). After 20-22 h of culture, COC and pieces of wall were washed and transferred into the same maturation medium but without hormonal supplements for another 20-22 h of culture. After IVM oocytes were fertilized in vitro and embryos were cultured by standard protocols (Stokes P. et al., Developmental Biology, 284, p.62 – 71, 2005). All chemicals used in this study were purchased from Sigma-Aldrich (Moscow, Russia). The question was: have $BCB^+$ oocytes from follicles of different diameters the same developmental competence? We did not find significant differences between the level of cleavage and blastocyst in all groups of experiments. Percentages of cleavage and blastocyst in groups were: follicles d < 3 mm - 43% (27/63) and 29% (18/63); follicles d 3 - 6 mm - 46% (45/98) and 35% (34/98); follicles d > 6 - 48% (28/58) and 28% (16/58) ($\chi^2$ test). Immunocytochemical analysis was used for detection of *estrogen receptor* expression (ER) in cumulus cells of 53 $BCB^+$ and 33 $BCB^-$ oocytes. Immunocytochemical staining was performed using the first rabbit polyclonal anti-human ER antibodies (NCL-ERp, Novocastra, OOO BMS, St.Petersburg, Russia). The visualization system (ABC-universal kit) consists of avidin–biotinilated peroxidase (DakoCytomation) was applied. 3,3'-diaminobenzidine was used as it was recommended from manufacture Novocastra (OOO BMS, St.Petersburg, Russia). Hematoxylin *(Abrisplus,* St.Petersburg, Russia) was used to stain cells. Antigen optical density was measured using morphometric VideoTest (Russia) computer program. Positive immunocytochemical reaction was mainly observed in the nuclei membrane and slightly on the cytoplasmic membrane of cumulus cells (probably as non-specific background). It was shown that cumulus cells of $BCB^+$ oocytes had a significantly more pronounced expression of the ER than the cumulus cells of $BCB^-$ oocytes ($p < 0,001$, Mann-Whitney test).

This study was supported by grant No.14-04-90038 Bel_a from Russian Foundation of Basic Research.

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# Determining intrafollicular concentrations of cortisol and progesterone in horses and the effects of cortisol on *in vitro* maturation of equine oocytes

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**Keywords**: cortisol, follicles, horse, progesterone, IVM, oocytes.

Increased glucocorticoid release and synthesis in response to acute or chronic stress has been shown to impair reproductive function in a variety of species and therefore may affect fertility. The aims of this study were 1) to determine cortisol and progesterone concentrations in equine follicular fluid and serum and 2) to assess the effects of cortisone supplementation to the maturation medium on IVM rates of equine oocytes. We hypothesized that challenging equine oocytes during IVM with higher doses of cortisone than physiological levels does not affect IVM rates. Light horse mares (n=9) used in this study were reproductively sound and cycling. Follicular fluid samples were collected by ultrasound-guided transvaginal follicle aspiration from the following follicle classes: G1: 5-9 mm, G2: 10-14 mm, G3: 15-19 mm, G4: 20-24 mm and G5≥25 mm. Blood samples were collected from each animal at the beginning and at the end of the aspiration period, respectively. Hormone determinations for cortisol (DE1887, Demeditec, Kiel-Wellsee, Germany) and for progesterone (ADI-901-011, Enzo Life Sciences, Farmingdale, NY, USA) were performed by ELISA. Cumulus oocyte complexes (COCs) were collected by OPU only from healthy, growing follicles, in the absence of a preovulatory follicle. Compact COCs (n=84) were randomly assessed either to control group, or to one of the treatment groups, in which hydrocortisone (H4001, Sigma Chemical, St. Louis, MO, USA) was added to the standard maturation medium in the following concentrations: 0.1 µg/ml, 1 µg/ml, 5 µg/ml and 10 µg/ml. After 30h, oocytes were denuded, stained with Hoechst (33342, Sigma) and IVM rates were assessed. Statistical analysis was done with the SPSS Statistics 22 software. As all data were normally distributed (Kolmogorov–Smirnov test, $p > 0.05$ for all parameters), one way ANOVA, Post-Hoc-Test and Pearson's correlation were applied for the hormones, whereas Chi-Square Test was used to analyse IVM rates. In follicular fluid from G5 follicles, concentrations of cortisol and progesterone were significantly higher ($p<0.05$) than in all other groups. Concentrations of cortisol and progesterone were positively correlated ($r=0.8$; $p<0.001$). In contrast, serum concentrations of progesterone and cortisol in mares did not differ at the beginning and the end of the aspiration period. There was no significant difference in the percentage of matured oocytes between groups, regardless of the concentration of cortisone added to the medium. Our results demonstrate a significant increase of cortisol in preovulatory follicles in vivo, suggesting its importance for oocyte maturation. Moreover, challenging equine oocytes in vitro with up to 100 times more cortisol than physiologically existent in follicles larger than 25 mm did not significantly affect IVM rates, suggesting that the equine oocyte is able to modulate cortisol levels and therefore to adapt to stress situation.