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The estrogen and progesterone receptors in porcine cumulus cells during real-time cell proliferation

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Keywords: estrogen, progesterone, pig.

The expression of estrogen and progesterone receptors within porcine ovary and cumulus-oocyte-complexes (COCs) is well recognized, but still little is known about expression of progesterone receptor (PGR), PGR membrane component 1 (PGRMC1) and of estrogen-related receptors (ERRγ and ERRβ/γ) in separated cumulus cells in relation to real-time proliferation.

In this study, COCs were tested by brilliant cresyl blue (BCB) test (Sigma-Aldrich, St. Louis, MO, USA). Only BCB-positive oocytes were used. The cumulus cells (CCs) were separated from COCs and were used to analyze the cell proliferation index (CPI) and the expression PGR, PGRMC1 and of ERRγ and ERRβ/γ during a 96h cultivation *in vitro* using RT-qPCR and confocal microscopic observation. CPI was evaluated at four steps of cultivation (0-96h, 0-9h, 8-62h, 58-96h). The rabbit polyclonal antibodies anti-PGR, anti-PGRMC1, anti-ERRγ and anti-ERRβ/γ (Santa Cruz Biotechnology, Santa Cruz, CA, USA), were applied. Then, CCs were stained with 0.1 μg/ml 4,6-diamino-2-phenylindole (DAPI; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Confocal microscopic images were analyzed using Imaris 7.2 software (BitPlane, Zurich, Switzerland). For statistical analysis one-way analysis of variance (ANOVA) and Tukey test, were applied. These tests were used to compare the results of real-time quantification of the proliferation index.

We found that PGR protein expression was increased at 0h, compared with PGR protein expression after 96h of culture ($P < 0.001$). The expression of PGRMC1, ERRγ and ERRβ/γ was unchanged. After using RT-qPCR we did not find significant differences in expression of PGR, PGRMC1, ERRγ and ERRβ/γ during 96h of cumulus cells in vitro culture.

We suppose that the different expression of the PGR protein at 0h and after 96h is related to a time-dependent down-regulation, which may activate a negative feedback. The distribution of PGR, PGRMC1 proteins may be linked with the translocation of receptors to the cytoplasm after the membrane binding of respective agonists and intra-cytoplasmic signal transduction. Furthermore, cumulus cells analyzed at 0h were characterized by decreased proliferation index, whereas those after 96h of culture revealed a significant increase of proliferation index, which may be associated with differentiation/luteinization of these cells during real-time proliferation.

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Inhibins expression in porcine oocytes isolated from follicles of different size

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Keywords: INHßA, INHßB, oocytes.

Inhibins are members of transforming growth factor beta (TGF-ß) superfamily. It was previously suggested that inhibin ßA (INHßA) as well inhibin ßB (INHßB) may be involved in the regulation of important stages of the growth of follicles and oocytes. Their function is mostly related to hormonal regulation of reproductive process as they down-regulate FSH synthesis and inhibit FSH secretion. Regulation of feedback loops between the pituitary and ovary, as well as their influence on folliculogenesis, has been shown previously. On the contrary there is a limited number of reports describing the role of follicle size during oocyte maturation. This study was aimed to investigate differential expression of INHßA and INHßB in porcine oocytes before and after *in vitro* maturation (IVM) isolated from follicles of various sizes.

The ovaries and reproductive tracts were recovered from gilts immediately after slaughter and transported to the laboratory. Follicles were aspirated by individual puncturing. Cumulus-oocyte complexes (COCs) were selected under an inverted Zeiss microscope (Axiovert 35, Lübeck, Germany). Only COCs with homogenous ooplasm, uniform and compact cumulus cells were used. The developmental stage of collected COCs was investigated by brilliant cresyl blue (BCB) test (Sigma-Aldrich, St. Louis, MO, USA). Porcine oocytes (each n = 40) were isolated from large (>5mm), medium (3-5mm) and small (<3mm) follicles, and were used to study the INHßA and INHßB protein expression pattern using Western blot analysis before and after 44h of oocyte IVM. The proteins expression levels were evaluated using densitometric analysis (GelDoc iT Imaging System, Eppendorf). The analysis of variance (ANOVA) and Tukey test, were used for statistical analysis.

We observed an increased expression of INHßA in oocytes collected from large and medium follicles compared to small follicles before IVM ($P < 0.001$, $P < 0.001$). After IVM, expression of this protein was higher in oocytes isolated from large follicles compared to medium and small follicles ($P < 0.01$, $P < 0.001$). Similarly, higher INHßB levels were observed in oocytes recovered from large follicles compared to small before IVM ($P < 0.01$) and after IVM ($P < 0.01$).

Since INHßA and INHßB are expressed in both porcine follicular somatic cells and oocytes, it may be assumed that these TGF-ß superfamily factors are involved in the regulation of molecular bi-directional pathways during follicle and oocyte development. It has also recently been shown that inhibins may act as regulators of oogenesis and could be markers of the developmental potential of oocytes. As in our study expression levels of INHßA and INHßB were higher in medium and large follicles. We assume that this correlation can be a marker of higher maturational competence of oocytes of larger size.

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Brilliant Cresyl Blue selection of cat cumulus-oocyte complexes does not improve nuclear maturation following IVM

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Key words: cat, cumulus-oocyte complexes, Brilliant Cresyl Blue, maturation.

Some felid species, such as the Iberian Lynx, are among the most endangered species, and the use of Artificial Reproductive Techniques may constitute a valuable tool for recovering wild populations. *In vitro* maturation (IVM) in these species poses a challenge, as many undergo inducible ovulation following mating and therefore may not respond well to conventional IVM techniques used in other domestic animals exhibiting cyclic ovulation. The domestic cat provides a good model for endangered felids, as ovaries are available from sterilizations. As in other felids, oocyte maturation rates in cats remain low following conventional IVM techniques. Brilliant Cresyl Blue staining (BCB) is a dye that can be degraded by the enzyme G6PDH. BCB has been successfully used to select for developmentally competent oocytes in several cyclic ovulatory species: competent oocytes exhibit low G6PDH activity, being BCB+ as they are unable to degrade the dye, in contrast to the less competent BCB- oocytes. The objective of this experiment has been to determine whether BCB selection improves oocyte maturation rates in cat oocytes. As a preliminary experiment we tested the protocol in bovine oocytes by incubating cumulus-oocyte complexes (COCs) in PBS supplemented with 52 μM BCB for 90 min prior to IVM. Following IVM, the cumulative percentage of blastocysts 9 days after IVF were significantly higher in BCB+ oocytes (35.3 ± 3.8 vs 17 ± 1.4%, mean ± standard error of the mean –s.e.m.-, 5 independent replicates, 621 COCs, ANOVA $P < 0.05$), as expected. Then, we tested different IVM media supplementations in cat COCs observing that the percentage of oocytes exhibiting a metaphase plate improved with the addition of either 10 ng/μl of EGF (Epidermal Growth Factor) or 0.02 IU/ml FSH (Follicle-stimulating Hormone) + 0.01 IU/ml LH (Luteinizing Hormone) to BSA supplemented TCM-199 medium (43.7 ± 3.6 vs 64.7 ± 7.2 vs 62 ± 5.9 % for not supplemented, EGF or FSH + LH supplemented media, respectively, ANOVA $P < 0.05$, 3 replicates, 265 COCs). Finally, we tested whether BCB selection following incubation for 90 min in PBS supplemented with 52 μM BCB improved nuclear maturation following IVM in TCM-199 supplemented with EGF as above. The percentage of oocytes exhibiting a metaphase plate was similar in the BCB+ and BCB- groups (58.9 ± 4.6 vs 54.4 ± 3.6 %, respectively, 4 replicates, 305 COCs). In conclusion, BCB does not select for cat oocytes with a higher nuclear maturation ability.

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The influence of gonadotropin stimulation on breeding behavior in dairy cattle heifers

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Keywords: breeding behavior, superovulation, hormones.

The objective of the study was to determine the influence of gonadotropins on breeding behavior in dairy cattle. Therefore we investigated the breeding behavior in group housed German Holstein heifers during natural estrus (NE) followed by a superovulation treatment (ST). In this study we used eight heifers with an average age of 17.5 month (range 15.7 to 19.1 month). For NE blood samples to measure progesterone and estradiol levels and clinical and ultra-sonographic examinations were performed daily from day 18 after the previous estrus. For ST these examinations took place at the day of the first gonadotropin administration. The examinations for both treatments were continued till the day of ovulation. ST includes eight intramuscular injections of 800 IU of Follitropin and Lutropin (PLUSET®, Laboratorios Calier S.A., Spain) on four consecutive days with descending dosage and two intramuscular injections of 0.5mg Cloprostenol (PGF VEYX® Forte, Veyx-Pharma GmbH, Germany) on day three. Estrus behavior for each heifer was assessed by video-analysis continuously for three days in the periestrus- and estrus-period (day -1: before estrus, day 0: behavioral estrus, day 1: after estrus) using the software Observer XT 10 (NOLDUS, Wageningen Netherlands). The following behavior traits were recorded: Sniffing, chin-resting, mounting, mounting head side and standing heat. Breeding behavior was summed up for six hours intervals and rated according to the scale suggested by Van Vliet and Van Eerdenburg (VanVliet and VanEerdenburg, Applied Animal Behaviour Science 50, 57-69, 1996). Six hours intervals with more than 1200 estrus score points were considered as estrus interval and summed to calculate estrus length. The statistical analysis was performed with SigmaPlot 12 using Wilcoxon Signed Rank Test.

Our results show hardly any difference between the two groups. We found a significant difference in progesterone levels on day one after estrus. In the ST the animals showed higher progesterone levels ($P = 0.016$, Median: 0.565ng/ml) compared to the NE (Median: 0.240ng/ml). There was no statistical significant difference for estradiol. The expression of breeding behavior traits were not statistical significant between NE and ST, because of high variations in intra-individual estrus behavior. There was no statistical significant variation in estrus length, although three of eight heifers showed a longer duration but none a shorter duration of estrus behavior in ST (average 19,5h) compared to NE (average 15,75h).

These results show that there are slightly any differences in breeding behavior between the two treatments. For a better characterization of sexual related behavior and the influence of hormones further studies are needed including other behavior traits (e.g. vocalization) and hormone profiles.

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Nuclear magnetic resonance (NMR) of goat follicular fluid shows different metabolic profiles among follicle size and female age

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Keywords: NMR, follicular fluid, metabolic profile.

Oocytes recovered from prepubertal goats are highly heterogeneous in growth and grade of atresia which make them unpredictable for IVEP programs. In our laboratory we have observed that oocytes from 2 month-old goats obtained from > 3 mm follicles develop up to blastocyst stage at a similar percentage than oocytes from adult goats (18% and 21%, respectively), suggesting that the follicle development and the follicular fluid (FF) content are more relevant to oocyte competence than the age of the donor (Romaguera, Theriogenology, 76(1), 1, 2011). The aim of this study was to characterize the FF metabolomic profile from different follicular environments. A High-resolution proton nuclear magnetic resonance (^{1}H NMR)-based metabolomic study was carried out. Samples of adult (n = 40) and prepubertal (n = 16) FF were collected by LOPU and aspiration of slaughterhouse ovaries, respectively. FF from small (< 3 mm) and large (> 3 mm) follicles were pooled for each female. 1D ^{1}H NMR experiments were done on a Bruker AVANCE 600 spectrometer (BrukerBiospin, Rheinstetten, Germany; 600.13 MHz). Sample handling was controlled with TOPSPIN 3.1 software. Signals were assigned to their metabolite by comparing resonance frequencies (expressed as ppm) and line shapes to prior data. Multivariate ordination principal component analysis (PCA) was done to detect patterns of sample ordination. The AMIX 3.9.14 software package was used to process the ^{1}H NMR spectra and perform the statistical analysis with the significance testing approach described by Goodpaster (Anal Biochem, 401, 134, 2010). The unsupervised method clearly differed between the FF metabolomes of large and small follicles of prepubescents. The variables responsible for the discrimination were inositol (3.63 and 3.53 ppm) and lysine (3.78 ppm) which presented higher concentrations in small follicles ($P < 0.001$, Bonferroni corrected confidence interval). When large and small follicles were considered together, a significant difference between adult and prepubertal metabolic profiles was observed by visual comparison of the spectra, corresponding to the presence of α,β-glucose (3.43, 3.88, 3.48, 3.73, 4.68, 5.33 and 5.28 ppm) in adults and absence in prepubescents. Other metabolites differed significantly between the two groups ($P < 0.001$, Bonferroni corrected confidence interval): lactate (4.13 and 1.33 ppm), N-CH3 groups (3.23 ppm) and inositol (4.08 ppm), which were higher in prepubescents. In conclusion, these results showed that metabolomic profiles are different according to the follicle diameter and the female age. Some of these metabolomes could be related to the acquisition of oocyte competence and might be used as biomarkers of oocyte quality.

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Formation of cystic ovarian follicles after intrafollicular injection of indomethacin prior to ovulation in heifers

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Keywords: Indomethacin, cystic follicles, cattle.

The mechanisms for cystic ovarian follicle (COF) formation in cows are not fully understood. In this study we aimed the use of the cyclooxygenase (COX) inhibitor Indomethacin via intrafollicular injection to induce artificial COF in cattle. Preovulatory follicles (POFs) were produced in Holstein-Frisian heifers by the administration of 0.5 mg of Cloprostenol im (PGF; Veyx forte, Veyx-Pharma) in diestrus followed by 0.1 mg Gonadorelin im (Gonadovet, Veyx-Pharma) 54 h later. At 70 h ultrasound-guided transvaginal intrafollicular injections in the POFs were performed as described previously by Vernunft et al. (Proceedings of AETE, 2014, p 184). In the first trail 279 µM indomethacin was injected and 1% ethanol solutions as vehicle control (0.2 ml, n = 5 for each group) in POFs. Follicle development was monitored by daily ultrasound examinations. Blood samples were analysed for progesterone levels biweekly by a H3-RIA assay. In the second trail decreasing concentrations of indomethacin (0.2 ml of 70, 35 and 5µM; n = 4 for each concentration) were injected to find the minimal dose for ovulation prevention and COF formation as monitored by ultrasound examinations. In the third trail injections with 0.2 ml solution of 60 µM NS398 (COX2 inhibitor) or 280µM SC560 (COX 1 inhibitor) in POFs were performed unravel to investigate the manly involved COX pathway (n = 4 for each specific inhibitor) and controlled ovulation 24 h later. Data are presented as means ± SD and differences between groups were determined by t-test ($P < 0.05$). In the first trail intra-follicular indomethacin injections inhibited ovulation in all animals while all the controls ovulated. The diameter of the injected follicles was 14.9 ± 0.7 mm at PGF administration, 17.8 ± 0.7 mm prior to the follicle injections and 34.6 ± 0.7 mm 7 days later. In the non- ovulated follicles, starting from day 5 after follicle injection an increasing thickness of the follicle wall and vascularization was observed during the (Doppler-) ultrasound examinations, indicating the beginning of luteinisation. Plasma progesterone levels on day 7 after intra-follicular injections were significantly lower in the indomethacin treated group than in the controls (1.5 ± 0.7 ng/ml vs. 3.2 ± 0.1 ng/ml, $P < 0.05$). The injections of 70 µM and 35 µM indomethacin solutions in the second trail also inhibited ovulation and led to COF formation, while 5µM solutions inhibited ovulation only in two out of four heifers. However, neither injections of the specific COX 1 nor the COX 2 inhibitor inhibited ovulation in the third trail. In conclusion, intra-follicular injections of 0.2 ml 35 µM indomethacin solution led to COF formation in heifers. Presumably, indomethacin does not act only via the cyclooxygenase pathway in the POFs.