performing the second insemination, it was difficult or impossible to penetrate cervical canals. Thus, only 1 tahr became pregnant. These findings show 13 days of CIDR insertion, the dose of PMSG 400 IU and hCG 200 IU, and the use of PGE2 as beneficial for inducing estrus synchronization, and prove that extended-chilled semen may also be used for artificial breeding of Himalayan tahr. This is the first report of successful pregnancy induced by artificial insemination of fresh or extended-chilled semen in oestrus-synchronized Himalayan tahrs.

**Folliculogenesis/Oogenesis**

161 DIETARY ENERGY SOURCE IN PRIMIPAROUS DAIRY COWS DURING THE TRANSITION PERIOD: BLOOD METABOLITES AND FOLLICULAR CLASSIFICATION

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Solving reproductive problems of postpartum dairy cows has become one of the main targets of the scientific community even though the advances in this field are partial in most of the conducted research. There is a need to approach the problem with a multidisciplinary strategy that simultaneously includes nutritional and reproductive issues. Increasing the availability of glucogenic and lipogenic nutrients during the transition period has been hypothesised to improve energy balance and to decrease the incidence and severity of metabolic and reproductive disorders in early lactation. Based on the considerations mentioned above, the objective of this study was to compare the effects of a glucogenic or a lipogenic diet on blood metabolites and follicular dynamics in primiparous dairy cows during the transition period. Forty primiparous dairy cows received a lipogenic or glucogenic supplement during the transition period and were randomly assigned to 1 of 4 treatments [control; calcium salts of soybean fatty acids (CaS), Megalac-E; toasted soybean; and propylene glycol]. Diets were isocaloric and isoproteic. Blood samples were taken from each animal 10 and 5 days before the expected calving date and at days 7, 14, 21, 28, 35, and 42 postpartum. Ultrasound scanning was performed in all cows on Monday, Wednesday, and Friday up to day 45 postpartum. Follicles registered during ultrasound examination were classified in 4 categories: class I (3–5 mm), class II (6–9 mm), class III (10–15 mm), and class IV (>15 mm). Data analysis was performed using PROC GLM (SAS, 6.12, SAS Institute Inc., Cary, NC, USA, 1995). Metabolites and ovarian activity were analysed using a split-plot design. Pairwise comparisons of individual means were carried out using the Scott Knott and Duncan test. Values of \(P < 0.05\) were considered statistically significant. Insulin and glucose concentrations were higher in the CaS group when compared with other treatments (\(P < 0.05\)). The lowest insulin and glucose concentrations were observed on cows supplemented with propylene glycol. Nonesterified fatty acid concentrations (NEFA) were lower for all treatments compared with the control group (\(P < 0.05\)). The most efficient follicular growth pattern during the postpartum period was observed in the CaS group, which was characterised by a reduction in the number of class I and II follicles and an increase in class III and IV throughout the trial. In general, smaller follicles go through recruitment and selection processes to bigger follicles and these follicles probably remain stable due to the fact that this particular follicular class represents a transitory phase in which there are always follicles going under the processes of selection and dominance. Overall, results suggest that calcium salts of soybean fatty acids reduced the dramatic metabolic and endocrine changes of primiparous dairy cows during transition period, which could be associated with better reproductive performance.

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162 OVARIAN ULTRASOUND BIOMICROSCOPY IN RABBITS

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Invasive methods (e.g. histology) limit serial assessment of ovarian structures in rabbits and impede progress in our understanding of factors responsible for inducing ovulation. Serial transrectal or transvaginal ultrasonography of ovarian structures has revolutionized our understanding of reproductive patterns in domestic animals, but the approach is limited in smaller species by the small physical size of the animal and the ovary. The advent of high-resolution ultrasound biomicroscopy (UBM), however, has permitted recent characterisation of ovarian dynamics in species as small as mice. As part of a larger study on factors influencing ovulation in rabbits, the objective of the present study was to validate UBM as a method of assessing ovarian structures in rabbits when comparing ultrasound image attributes with histological attributes. Female New Zealand White rabbits (\(n = 4\); 5 to 5.5 months of age) were given ovulation-inducing treatments and examined daily by conventional transabdominal ultrasonography with a 12-MHz linear-array probe (MyLab\(^\text{TM}5\)). Rabbits were killed with sodium pentobarbital 8 days after treatment. Ovarian UBM was performed \textit{ex situ} in a water bath. A 25-MHz oscillating sector-array transducer (Visualsonics) was moved mechanically at a constant speed along the long axis of each ovary, and images were recorded (300 frames per 10-s cine-loop). After UBM, ovaries (\(n = 8\)) were fixed in 10% formaldehyde, embedded in paraffin, serial-sectioned at 10-\(\mu\)m thickness, and stained with Masson’s trichrome. Digital photomicrographs were taken of every third section of each ovary. For each ovary, follicles \(\geq 0.6\) mm and corpora lutea (CL) were counted and measured by scrolling through the UBM cine-loops and the serial micrographs. Ovarian follicles \(\geq 1.5\) mm were readily detected by conventional ultrasonography, and such follicles were present in the ovaries of all rabbits on the day of treatment, but CL structures were not clearly discernable during the 8 days after treatment. \textit{In vivo} transabdominal UBM failed to yield satisfactory images of the ovaries probably because of high acoustic impedance of the body wall. The number (mean \(\pm\) SEM) of CL detected by UBM \textit{ex situ} in each pair of ovaries was highly correlated (\(r = 0.99\); \(P < 0.05\)) with the number detected by histology (8.5 \(\pm\) 2.9 vs. 8.8 \(\pm\) 3.0). The diameter of CL was also highly correlated (2.1 \(\pm\) 0.7 vs. 1.8 \(\pm\) 0.6 mm; \(r = 0.99\); \(P < 0.05\)). Similarly, a high correlation was detected between UBM and histology in the number of follicles \(\geq 0.6\) mm (17.3 \(\pm\) 2.3 vs. 19.0 \(\pm\) 1.6; \(r = 0.96\); \(P < 0.05\)), and follicle diameter (1.1 \(\pm\) 0.05 \(\mu\)m).
1.1 ± 0.03 mm; r = 0.96; P < 0.05). Interestingly, UBM of each ovary required on average 2 h compared with 30 h for histology. The results validate the accuracy of UBM for assessing rabbit ovaries ex situ, but identify limitations of present ultrasound approaches for examining rabbit ovaries in situ.

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### 163 IN VITRO CULTURE OF PORCINE OOCYTE-GRANULOSA CELL COMPLEXES

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The objective of this study was to investigate the effects of porcine follicular fluid (PFF) and insulin-like growth factor-1 (IGF-1) on the growth of prepuberal and adult cattle, a comparative study was conducted by measuring mRNA expression of 4 developmentally relevant, but non-imprinted genes about the mechanisms involved in attaining of the full developmental potential of bovine oocytes. Using immature and possible reason for this phenomenon. Particularly DNA methylation, allele specific gene expression in a parent-of-origin-specific manner (imprinting), achieving actual cultivation maturity. However, several studies proved that oocytes derived from prepuberal animals differ significantly from oocytes of 2nd lactation, either left untreated (Ad1) or treated with FSH (Ad2) [30 and 40.6% (10 and 50 ng mL\(^{-1}\) IGF-1), P < 0.05]. The maturation rate of oocytes grown with 10 and 50 ng mL\(^{-1}\) IGF-1 was higher than those of the other 2 groups [25 and 26% (0 and 100 ng mL\(^{-1}\) IGF-1, respectively); P < 0.05]. Thus, the second conclusion is that supplementation of culture medium with PFF did not enhance the development of porcine OGC in vitro. In part 2 of the experiment, treatment groups were cultured with 10, 50, or 100 ng mL\(^{-1}\) IGF-1. The percentages of OGC showing antrum formation were 80, 80, 100, and 100% in groups treated with 0, 10, 50, or 100 ng mL\(^{-1}\) IGF-1, respectively. Average oocyte diameter was 94.16 and 94.58 μm just after OGC collection. However, the average diameter of oocytes cultured for 12 days with 50 or 100 ng mL\(^{-1}\) IGF-1 was significantly higher than that of the control or 10 ng mL\(^{-1}\) IGF-1 groups [108.88 and 108.31 μm (50 and 100 ng mL\(^{-1}\) IGF-1) vs. 105.98 μm and 106.67 μm (0 and 10 ng mL\(^{-1}\) IGF-1), P < 0.05]. The maturation rate of oocytes grown with 10 and 50 ng mL\(^{-1}\) IGF-1 was significantly higher than those of the other 2 groups [25 and 26% (0 and 100 ng mL\(^{-1}\) IGF-1, respectively); P < 0.05]. Thus, the third conclusion is that 50 ng mL\(^{-1}\) IGF-1 improves the growth and maturation of porcine oocyte-granulosa cell complexes in vitro.

### 164 EPIGENETIC ANALYSIS OF GENOMIC DNA IN PREPUBERAL AND ADULT BOVINE OOCYTES

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The use of oocytes obtained from prepuberal cattle shortens the generation interval by producing descendants of genetically valuable animals before achieving actual cultivation maturity. However, several studies proved that oocytes derived from prepuberal animals differ significantly from oocytes of adult animals with regard to their development capability and therefore reproductive potential. Epigenetic events are taken into consideration as a possible reason for this phenomenon. Particular DNA methylation, allele specific gene expression in a parent-of-origin-specific manner (imprinting), and certain histone modifications, like acetylations, croxylations, and phosphorylations, play an important role. This project aims to gain knowledge about the mechanisms involved in attaining of the full developmental potential of bovine oocytes. Using immature and in vitro matured oocytes of prepuberal and adult cattle, a comparative study was conducted by measuring mRNA expression of 4 developmentally relevant, but non-imprinted genes (GDF9, GLUT1, PRDX1, and ZAR1) as well as the general DNA methylation status, performed by bisulite sequencing of 2 satellite sequences [bovine testsis satellite I DNA segment 2 (BTS2) and Bos taurus satellite I DNA (BTS)]. After various pretreatments, immature bovine oocytes were collected from prepuberal calves [6–9 months, either left untreated (Ca1) or treated with FSH (Ca2) or FSH+IGF1 (Ca3) or FSH+IGF1 (Ca4)] and adult animals [≥2nd lactation, either left untreated (Ad1) or treated with FSH (Ad2)] using the Ovum-pick-up (OPU) technique. The Ad1 group was considered the control group. First results of the qPCR analyses of immature oocytes show differences between treatment groups for GLUT1, PRDX1, and ZAR1 transcripts. Compared with Ad1, GLUT1 expression increased in Ad2 [fold change (FC) 2.2], Ca1 (FC 2.0), Ca2 (FC 1.8), and Ca3 (FC 1.4). The genes PRDX1 and ZAR1 were reduced in all groups by 0.02 to 0.07 in comparison with Ad1. The GDF9 showed generally a very low expression. The methylation analysis shows for BPS2 and BPS significant differences before and after in vitro maturation in the groups Ad1 (BTS2: 49.6 ± 64.9%, Ad2 (BTS: 76.7 ± 52.5%), Ca1 (BTS2: 74.6 ± 53.3%), Ca2 (BTS: 72.8 ± 57.8%) and Ca3 (BTS2: 60.6 ± 71.7%). Currently, the first experiment and statistical analysis are underway. The preliminary data confirm differences in gene expression between prepuberal and adult animals, and demonstrates the dependence of the methylation pattern on age and maturation status. These results contribute to a better understanding of the developmental potential of prepuberal oocytes in order to optimize their use for in vitro production of embryos.

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165 SHORT-TERM FASTING AFFECTS METABOLIC MARKERS WITHOUT IMPACT ON FOLLICLE AND OOCYTE DEVELOPMENT IN THE RABBIT MODEL

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Our goal was to elucidate whether acute energy restriction has any influence on ovarian reproductive processes and the relationship with some metabolic markers in the rabbit model. A total of 22 nulliparous rabbits were randomly distributed in 2 groups: control group fed ad libitum (C; \( n = 12 \)) and 72-h fasted group (F; \( n = 10 \)). Before and after the deprivation period, body weight (BW), estimated body composition, serum triglycerides (TG), nonesterified fatty acids (NEFA), and leptin concentrations were measured. After fasting, ovaries were retrieved: one was used to study oocyte in vitro nuclear and cytoplasmic maturation (in terms of granule cortical migration; Arias-Alvarez et al. 2010 Theriogenology 73, 26–35) and the other one was processed histologically to assess follicle categorization and atresia rate by TUNEL and by immunolocalization of proapoptase-3 (PC3). Statistical analysis was carried out by MIXED and CATMOD procedures of SAS program. The BW of control rabbits increased at the end of the experiment (3.96 ± 0.06 v. 4.03 ± 0.09 kg; \( P < 0.05 \)), but the fasted group showed similar weight compared with 72 h before and the C group (3.97 ± 0.09 kg). No differences were observed on estimated body composition between C and F animals (61.0 ± 0.52% water, 2.96 ± 0.02% ash, 18.6 ± 0.51% lipids, 18.4 ± 0.05% protein, and 1206 ± 21.6 kJ/100 g of energy). The TG was similar between groups (74.4 ± 9.9 v. 49.3 ± 10.4 mg dl\(^{-1}\)), whereas NEFA concentrations were significantly higher in the F group than before fasting (0.29 ± 0.04 mmol L\(^{-1}\); \( P < 0.05 \)) and than in the control group (0.09 ± 0.04 mmol L\(^{-1}\); \( P < 0.05 \)). In opposition, leptin concentrations were lower in the F group than before fasting (2.2 ± 0.6 v. 5.1 ± 0.4; \( P < 0.05 \)) and than in C rabbits after fasting (4.6 ± 0.5 ng ml\(^{-1}\); \( P < 0.05 \)). No differences were found in the number of primordial (192.0 ± 52.9 v. 276.0 ± 88.6), primary (36.7 ± 6.6 v. 45.5 ± 7.8), secondary (37.1 ± 7.5 v. 54.1 ± 14.6), and antral follicles (21.9 ± 2.1 v. 22.3 ± 2.2) between the C and F group. The rates of healthy (53.2 ± 4.3 v. 57.4 ± 5.0), early atretic measured as <50% of apoptotic cells (15.4 ± 2.2 v. 17.2 ± 2.6), and late follicles measured as >50% of apoptotic cells (31.4 ± 3.6 v. 25.4 ± 4.2) did not differ between groups. Cytoplasm staining of PC3 was observed in stroma, follicles of all sizes, and corpora lutea of both groups. Atretic follicles did not exhibit immunoreactivity to PC3. No significant differences were observed in nuclear maturation and migrated cortical granule rates (63.6 ± 4.2 v. 4.7%, respectively). In conclusion, acute fasting affects BW and some metabolic markers (NEFA and leptin), but follicle and oocyte development is not impaired in our rabbit model.

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166 THE DIFFERENTIAL TRANSCRIPTOME AND ONTOLOGY PROFILES OF MURAL AND CUMULUS GRANULOSA CELLS IN STIMULATED HUMAN ANTRAL FOLLICLES

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Communication between various cell types in the ovary is a prerequisite for successful folliculogenesis and ovulation. In human antral follicles, granulosa cells divide into 2 distinct cell populations: mural (enveloping the antrum, MGC) and cumulus granulosa cells (surrounding the oocyte, CGC). During infertility treatment using in vitro fertilization (IVF), granulosa cells can be retrieved during puncture of stimulated follicles offering an excellent opportunity for analysing their functional properties. The aim of this study was to compare the transcriptomes of MGC and CGC. Twenty infertile women undergoing IVF-ICSI treatment were enrolled. The MGC were obtained from follicular fluid and CGC were acquired after oocyte denudation before micromanipulation. Gene expression of both cell populations was analysed using a genome-wide transcription array. The expression profile of the 2 granulosa cell populations varied significantly: out of 28,869 transcripts, 4,480 were differentially expressed (q-value < 10\(^{-4}\); 623 transcripts differed in their expression levels by at least 2-fold. The transcriptome of MGC showed higher expression of genes involved in immune regulation (toll-like receptors, IL18, IL17R). In CGC, pathways participating in intercellular interactions, tissue remodelling and protein degradation were more clearly distinguished (tenascin C, IGFBP5). Among the identified differentially expressed genes, several are involved in follicle development, oocyte maturation, or ovariolytic processes. Our findings fit well with previously published data. The results provide a basis for future studies on intra- and intercellular signaling in the preovulatory follicle leading towards identifying methods for improving oocyte health, embryo selection, and ultimately IVF success rate.
167 VISUALISATION OF FAT ACCUMULATION IN BOVINE PREANTRAL AND ANTRAL OOCYTES USING 2-PHOTON MICROSCOPY

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Fat metabolism is important in oocyte development (Kim et al. 2001; Sturmay et al. 2009). Our main objective was to develop a straightforward method that allows us to describe fat distribution and quantify fat accumulation in bovine oocytes throughout folliculogenesis. Fat accumulation dynamics can be studied by making microscopically repeated cross-sections of Z-stacks through oocytes of different developmental stages. The most important limiting factor in oocyte 3D visualisation studies is the size of the oocyte (between 110 and 120 µm in diameter), making it impossible to visualise it as a whole. If visualisation of the bottommost hemisphere is desirable, overlying Z-stacks will disperse the excitation and emission light, creating artefacts that will distort the image and trouble the results (indicated by former unpublished results). Therefore, we investigated the use of an alternative visualisation protocol staining the oocytes with Nile Red as an intracellular, triglyceride-specific (when emission is captured at 590 nm), fluorescent dye (Greenspan et al. 1985; Leroy et al. 2005), this combined with 2-photon excitation technology. In total, oocytes from 10 cows were collected at slaughter. Ovaries were pooled per cow. Only cows with apparent follicular activity on both ovaries were selected. Antral follicles with a diameter of <3 mm, between 3 and 6 mm, and >6 mm were aspirated. Apart from oocytes collected from each of these 3 follicle classes, preantral follicles were harvested from ovarian cortex tissue through mechanical isolation and enzymatic digestion by collagenase type IA. In total, 3 oocytes per follicle class were collected for each pair of ovaries. They were fixed in 2% glutaraldehyde and 2% formaldehyde and stained with Nile Red (1 µg mL\textsuperscript{-1}). After mounting, images were acquired with a Zeiss LSM 510 reversed 2-photon microscope (Carl Zeiss GmbH, Jena, Germany) using an excitation wavelength of 807 nm, with an emission spectrum covered between 580 and 591 nm. Our results show that even with biphoton excitation, it remains very difficult to visualise fat droplet distribution in >100-µm oocytes. However, accurate images can be obtained of the upper hemisphere of the oocyte. These images can be used in future research on the dynamics of the distribution and the accumulation of lipid. Preliminary descriptive results clearly show that the relative amount of lipid droplets is lower and their size is smaller in oocytes from preantral follicles compared with antral counterparts. We can preliminary confirm that fat accumulation and the aggregation in droplets might take place preceding or even during antral development. We can visualise preantral oocytes as a whole in contrast to antral oocytes where only the upper hemisphere is visible without distortions. Image analysis software is currently applied to allow for a more quantitative interpretation.

D. De Rijck.

168 CHARACTERIZATION AND DIFFERENTIATION INTO OOCYTE-LIKE CELL MASSES OF PORCINE MESENCHYMAL STEM CELLS DERIVED FROM OVARIAN THECA CELLS

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Recent findings have shown that ova#res after birth have germ line stem cells, which were considered as an alternative for the production of an animal model. The present study was therefore aimed to characterise ovarian theca cells and generate oocyte-like cell masses in vitro in porcine. Theca cells isolated from ovarian follicle were cultured in A-DMEM supplemented with 10% FBS at 38.5°C in a humidified atmosphere of 5% CO\textsubscript{2} in air. The cells were evaluated in a quantitative expression of transcription factors (Oct3/4, Nanog, and Sox2) by immunocytochemical staining and RT-PCR, and followed by differentiated into osteocytes, adipocytes, and chondrocytes under controlled conditions. Differentiation of multiple mesenchymal lineages was confirmed by RT-PCR and specific marker staining. Differentiated cells into osteocytes, adipocytes, and chondrocytes were characterised by von Kossa and Alizarin Red staining, Oil red O staining, and Alcian Blue staining, respectively. The specific genes of osteocytes (Osteocalcin, Osteocalcin, and Runx2) and adipocytes (aP2) were analysed by RT-PCR. In vitro oogenesis was induced in DMEM/F12 by the previously described method (Dyce et al. 2006) for 48 days. Expression of transcriptional factors (Oct4, Sox2, and Nanog) and oocyte-specific markers (c-Mos and GDF9b) was detected by RT-PCR in these differentiated cells. At 48 days of differentiation, the oocyte-like cell mass#s were further cultured in TC199 supplemented with 0.5 µg mL\textsuperscript{-1} FSH and 0.5 µg mL\textsuperscript{-1} LH for 15 days. Induced cells were morphologically observed following Hoechst 33342. Expression of Oct3/4 was detected by immunocytochemical staining in these cells. Among the transcriptional factors, only Sox2 was detected by immunocytochemical staining and RT-PCR in the theca cells. Differentiation to osteocytes, adipocytes, and chondrocytes was confirmed by specific-marker staining and gene expression by RT-PCR, respectively. The morphology of oocyte-like cell masses was distinct by 40 days of differentiation. Granulosa or cumulus-like cells were distributed through the whole surface of oocyte-like cell masses. Transcriptional factors, c-Mos, and GDF9b were detected in the cell masses by RT-PCR. After being transferred oocyte-like cell masses to TC199, zona pellucida-like structure
was formed around the edge of the cell mass. After 15 days of culture in TCM-199, the morphology of cells was changed into blastocyst-like structure, which surrounded cumulus-like cells. Oct3/4 was expressed by immunocytochemical staining in a blastocyst-like structure. These observations demonstrated that ovarian theca cells have similar characteristics to mesenchymal stem cells in view of multilineage differentiation. Theca cells can be differentiated into oocyte-like cell masses, which expressed oocyte-specific markers. These cell masses were further developed to a blastocyst-like structure, which expressed Oct3/4. Further studies are required to evaluate in vivo differentiation to oocyte-like cells.

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169 CHANGES OF PROTEIN PROFILES DURING FOLLICLE DEVELOPMENT AND IN VITRO OOCYTE MATURATION IN THE PIG

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When fully grown oocytes are removed from their follicles, they can resume meiosis and mature spontaneously under in vitro conditions. However, nuclear maturation under in vitro conditions is not accompanied by complete cytoplasmic maturation, which is essential for successful fertilization and the initiation of zygotic development. In the first study, to investigate protein patterns during oocyte maturation in vitro, immature oocytes (germinal vesicle stage; GV stage) as control and oocytes matured (M-II phase) in vitro were analysed. The porcine oocytes in the GV stage were put in culture with TCM-199 for 44 h for M-II-stage oocytes. Total proteins were extracted from 1200 oocytes for GV and M-II stages, separated on 2-D gels, and stained with silver. In general, the overall protein staining pattern between the 2 gels was remarkably similar for most protein spots. Analysis of the gels identified proteins that were up- or down-regulated between GV and M-II stages. Up-regulated proteins were identified as PDE4D, GPKOW, PG5M, HSP70, ZPG4, galk1, GST-β, PDX1, PDX2, and PDX3. In contrast, down-regulated proteins were identified as PRKAB1, GRP78, TD-pozi, ERP57, MPP1, DTNA, ZP3B, HSP90, HSP86, and HSP27. This study has identified a novel protein, named myomegalin, that interacts with cyclic nucleotide phosphodiesterase (PDE4D). The second study analysed changes in proteins in follicular fluids during porcine follicular development. Follicular fluids were collected from follicles of 1- to 2-, 2- to 6-, and 6- to 10-mm diameter from ovaries of slaughtered pigs. Total proteins were extracted from follicular fluids by M-PER Mammalian Protein Extraction Reagent. Differentially expressed proteins were analysed by MALDI mass spectrometry and searched on NCBInr. As a result, Spot No. 28 from the 2- to 6-mm follicle was Ig lambda chain C region, and Spot No. 32 and 33 from 6- to 10-mm were Apolipoprotein A-IV (APOA4). Increases of those proteins were correlated with follicular development. These results indicate that in vitro maturation changes the protein profile of porcine oocytes, which play important roles in the sequence of molecular events in porcine oocyte maturation and follicular development.

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170 NATURALLY OCCURRING CHRONIC MASTITIS COMPROMISES FOLLICULogenesis, AFFECTS VASCULARIZATION, AND INTERACTS WITH DIFFERENTIATION FACTOR GDF-9 IN BOVINE OVARIAN STROMA

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Recent studies have suggested an association between reproductive failure and mastitis in lactating dairy cows, but our understanding of how mastitis affects reproduction is still limited. In the present study we investigated the effects of naturally occurring chronic mastitis on the population dynamics of ovarian follicles. Ovaries and milk samples were collected from 74 cows at slaughter. Milk samples from each quarter, were analyzed following National Mastitis Council procedures. Based on the presence of major pathogens and somatic cell count results, animals were sorted in 9 groups, but only the 2 extremes were considered for further analysis: uninfected (n = 8) and affected by chronic mastitis (n = 9). Primordial, primary, and secondary follicles were counted and scored on similar surface area of ovary sections for each animal (mean ± SD = 5.65 ± 0.25 cm²). They were analyzed with Fisher’s exact test, and the association between health status and follicle number was estimated by odds ratios ± confidence limits. Vasculature area in the ovarian cortex of healthy and mastitic animals was identified using Bandeiraea simplicifolia-I lectin (BSL-1). Results were quantified with the dedicated software MacBiophotonics image1, NIH, USA, and subsequently analyzed with r-test for statistical significance. Follicles were further characterized by immunostaining with a GDF-9-specific antibody. The intensity of the staining was semi-quantified using a relative scale: 0, 1, and 2 for no, weak, and strong staining, respectively. Our results indicate no (P > 0.05) difference between the numbers of primordial and primary follicles in healthy and affected animals. In contrast, the number of secondary follicles was significantly lower in sick animals (odds ratio 10.50*, P < 0.05), indicating 10 times higher risk for a mastitic animal to have less than 2 secondary follicles per square centimeter. Ovarian stromal vasculature represented the 6.38 ± 0.66% of cortical area in healthy animals v. 4.24 ± 0.37% (P < 0.001) in affected cows. The GDF-9 immunostaining revealed decreased fluorescence intensity in mastitic animals. Our results show that chronic mastitis is associated with considerable alterations in follicle growth and differentiation with a decreased ability of primary follicles to develop into the secondary state in affected animals. This is accompanied by a significant decrease of ovarian vasculature and the down-regulation of the follicle differentiation-associated factor GDF. The present findings substantiate the hypothesis that mastitis can reduce fertility by exerting a negative effect on ovarian function.
172 REUSE OF AUTOCLAVED INTRAVAGINAL PROGESTERONE DEVICE TO ESTROUS SYNCHRONIZATION IN TOGGENBURG GOATS IN THE BREEDING SEASON

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Reusing intravaginal devices represents an important alternative to reduce costs; however, this practice may increase disease transmission. The aim of this study was to evaluate the efficacy of reusing autoclaved intravaginal progestrone devices for oestrous synchronization in Toggenburg goats in the breeding season was studied. This study was done in March and April of 2009, in Piauí, MG, Brazil (latitude 21°35’ and longitude 43°15’). Sixty-seven Toggenburg nulliparous (n = 17; 35.3 ± 5.4 kg and 3.3 ± 0.2) and pluriparous (n = 50; 52.9 ± 9.8 kg and 3.4 ± 0.3) goats were assigned according to weight and body condition score (BCS, 1 to 5 scale) into 3 treatments. Animals received new devices (n = 25; 48.2 ± 11.5 kg and BCS 3.4 ± 0.3) containing 0.33 g of progesterone (Eazi-Breed CIDR\textsuperscript{B}, Pfizer Animal Health, São Paulo, Brazil) or autoclaved (121°C, 1 atm, 15 min) devices previously used for 6 days (n = 23; 48.3 ± 13.0 kg and 3.5 ± 0.3) or 12 days (n = 22; 48.2 ± 11.0 kg and 3.4 ± 0.3). All goats received 5 mg dinoprost (Lutalyse\textsuperscript{E}, Pfizer Animal Health) in the vulvar submucosa on the day of CIDR insertion (Day 0) and 200 IU eCG (Novormon 5000\textsuperscript{B}, Sintex Indústria Bioquímica, Buenos Aires, Argentina) 1 day before CIDR removal, also in the vulvar submucosa. The CIDR were removed on day 6, and goats were bred twice daily with fertile bucks at oestrous onset and 24 h later if they were still in oestrus. Parametric variables were analysed by ANOVA and SNK tests by the BioEstat\textsuperscript{C} program. Nonparametric variables were analysed using the chi-square test by the BioEstat\textsuperscript{C} program. The results are described as mean ± SD. Oestrous response and conception rates did not differ (P > 0.05) among goats treated with the new devices (75.0; 54.2%) or those previously used for 6 (81.8; 50.0%) or 12 days (71.4; 47.6%), respectively. Non differences were detected between nulliparous (82.3; 52.9%) and pluriparous (72.0; 50.0%) goats. The interval from device removal to oestrus and duration of oestrus were not different (P > 0.05) among animals receiving a new device (39.3 ± 15.8; 30.7 ± 16.6 h) or previously used devices for 6 (32.7 ± 11.5; 31.8 ± 7.3 h) or 12 day (40.8 ± 20.7; 32.8 ± 13.2 h) treatments, respectively, or between nulliparous (41.6 ± 14.9; 30.9 ± 13.9 h) and pluriparous (35.6 ± 16.8; 32.1 ± 12.8 h) goats. Since there were no differences detected in the evaluated variables among goats receiving reused autoclaved devices or new ones, it can be suggested that the autoclaving process did not affect the efficiency of reusing intravaginal progestrone devices for oestrous synchronization in Toggenburg goats in the breeding season. Probably, P4 concentrations in goats receiving reused autoclaved devices reached at least minimum concentrations to promote oestrus response, since a non-treated group would not show oestrus in this level of synchronization as in goats in this study. This technique can be a simple and valuable tool to reduce sanitary risks of disease transmission without altering fertility in goats.

CNPq, Pfizer Animal Health, Embrapa Goats, and Sheep Research, CAPES.

173 A COMBINED RECOMBINANT BOVINE SOMATOTROPIN/EQUINE CHORIONIC GONADOTROPIN PROTOCOL IN THE ZEBU BREED TABAPUA AND HOLSTEIN HEIFERS SUBMITTED TO OVUM PICKUP

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The aim of this study was to enhance ovarian follicular development and subsequent oocyte retrieval by the use of a combined equine chorionic gonadotropin (eCG) and recombinant bovine somatotropin (rbST) protocol, as well as to compare its efficiency between the Brazilian zebu breed Tabapuá (TAB) and Holstein (HOL) nulliparous heifers. In a changeover design, TAB (n = 16) and HOL (n = 12) were allocated to 1 of 2 protocols in 2 distinct periods. Two weeks before initiation of the D2 (Dominant follicles were ablated by transvaginal ultrasound-guided aspiration: Group 1: on day 0 (D0) a subcutaneous injection of 500 mg of rbST (Bootstir\textsuperscript{B}, Intervet, SP) and on D2 an intramuscular injection of 500 IU of eCG (Novormon\textsuperscript{B}, Schering-Plough-Intervet, SP); and control: vehicle only. Two days later (D4), ovum pickup (OPU) was performed and the ovarian follicle population was evaluated by ultrasound. Twenty days after the first OPU session, heifers were switched across treatments and were aspirated a second time, so that a total of 28 sessions were performed for each treatment. Main effects of period, breed, and protocol and their interactions on follicle numbers and oocyte yield were analysed through the GENMOD procedure of SAS (SAS\textsuperscript{B}, Cary, NC, USA) using the Poisson distribution option. Means were compared by orthogonal contrasts, and the probability value set at 0.05 for significance unless otherwise specified. There was an interaction effect between hormonal stimulation and breed (P < 0.05) on the number of aspirated follicles (2 to 10 mm in diameter) such that they were higher in TAB treated (41.4 ± 2.6) compared with control (29.9 ± 2.6) heifers and did not differ between the HOL heifers (15.5 ± 2.6 v. 14.4 ± 2.6 in treated and control heifers, respectively). Overall, follicle numbers were higher (P < 0.0001) in TAB (35.6 ± 1.84) than in HOL (15.0 ± 2.1) heifers and in treated (28.4 ± 2.0 v. control 22.1 ± 2.0) heifers (P < 0.001). There was no effect (P = 0.77) of treatment on total viable oocytes (grades 1 to 3) between treated (3.5 ± 1.2) and control (3.9 ± 1.1) heifers. Tabapuá heifers had more (P < 0.05) viable oocytes than HOL (5.9 ± 0.9 v. 1.6 ± 1.4). In conclusion, the proposed protocol may improve OPU results but viable oocyte yield was not associated to the higher number of follicles available for aspiration. It may be necessary to modify this protocol to improve results perhaps by increasing the time window between rbST and eCG injections as well as by increasing the rbST dosage for heifers. The present protocol may be cost saving, compared with follicle stimulating hormone, for OPU sessions in the case of Tabapuá heifers because it is expected that responses decrease after a sequence of eCG injections. It is also interesting to notice that it is possible that HOL heifers already have higher endogenous growth hormone concentrations.
which could help to explain the interactive effect observed in the ovarian response. Further studies are necessary to improve this protocol especially in HOL heifers.

**CNPQ, CAPES, Schering-Plough-Intervet.**

### 174 EVALUATION OF THE 9-DAY PROTOCOL FOR ESTROUS SYNCHRONIZATION IN SANTA INES EWES


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The objective of the present study was to characterise follicular dynamics in Santa Inés (SI) during a 9-day protocol for oestrous synchronization and to evaluate the efficiency of the vaginal progesterone-releasing device Primer-PR\(^{B}\) (Tecnopec, Brazil). Cyclic females were used (n = 10) at the ruminant sector of the veterinary hospital of the FMVZ-USP, in March 2009. The animals had body scores between 2.5 and 3 and were of proven fertility. Ultrasound examinations were performed using an ALOKA SSD-500 Scanner (Berger, Brazil) with a linear 5.0-MHz transducer, attached to a handle to allow safe intrarectal manipulation. Examinations were performed daily from 3 days (D-3) before the day of device placement (D0) until the day of device removal (D9), and twice daily from device removal until ovulation. All ewes received 0.03 mg of prostaglandin (D-cloprostenol, Prolise\(^{B}\), Tecnopec, Brazil) on D9. Ovulation was assessed by the disappearance of the growing larger follicle or follicles present in the previous examinations. Oestrous detection was performed using an intact male 3 times a day (at 10:00 a.m., 6:00 p.m., and 12:00 p.m.) from D9 until the last acceptant ewe, and blood samples were taken by jugular puncture for progesterone (P4) measurement by radioimmunoassay on solid phase (COAT-6.56, Charles River, USA) from D-3 until the day of ovulation. Data were analysed by Shapiro–Wilks (PROC UNIVARIATE) using the SAS program (SAS Institute Inc., Cary, NC, USA, 2001) and shown as mean ± standard deviation. Plasma P4 concentrations between D0 and D9 were 6.56 ± 2.32 ng mL\(^{-1}\), peaking between D3 and D5 (8.07 ± 2.31 ng mL\(^{-1}\)). Oestrous behaviour was shown 45.6 ± 12.71 h after Primer-PR\(^{B}\) removal. The first and the last that showed oestrous behaviour was at 30 h and 66 h after Primer-PR\(^{B}\) removal, respectively, and the majority of ewes (50%) at 42 h. Oestrous lasted 26.40 ± 9.47 h, and the majority of ewes (70%) showed oestrous behaviour during 24 h. Ovulation occurred 73 ± 14.38 h after Primer-PR\(^{B}\) removal and 1.3 ± 0.48 ovulations per animal were observed. From all growing presumptively dominant follicles observed, 92.3% of them ovulated. When double ovulations occurred (n = 3), the interval between first and second ovulation was 16 ± 9.3 h. Emergence of the ovulatory follicular wave occurred at 8.5 days ± 16 h after Primer-PR\(^{B}\) insertion. The follicles observed to continue growing had a diameter of 3.48 ± 0.28 mm when they were first detected and reached 5.63 ± 0.66 mm, with a growth rate of 0.73 ± 0.43 mm per day. A standard follicle wave within the 9-day protocol was not possible to determine, and the follicles receded in up to 4 days. In conclusion, placement of a Primer-PR\(^{B}\) device for 9 days resulted in synchronous oestrus and ovulation in Santa Inés ewes.

FAPESP, CAPES, Tecnopec.

### 175 METABOLIC STRESS IMPAIRS FOLLICULAR GROWTH IN SUPEROVULATED HEIFERS

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Metabolic stress during the early postpartum period has been postulated to be one of the major causes of prolonged calving intervals. To study the impact of metabolic stress on ovarian follicular function via hormonal analysis of follicular fluid and follicular growth, 12 cycling heifers were randomly allocated to a group (n = 6) that received a control diet (ad libitum grass silage) or to an experimental group (n = 6) that was fasted during the superovulation (SO) treatment for 4 days until ovariectomy (Jorritsma et al. 2003 Theriogenology 60, 151–161). Heifers were synchronized with a CIDR\(^{B}\) intravaginal device (Pfizer AH) during 7 days, and a prostaglandin injection (5 mL Enzaprosto\(^{B}\), CEVA AH) was administered 1 day before CIDR\(^{B}\) removal. On day 9 of the synchronized cycle, the dominant follicle of the first follicular wave was removed and the standard SO protocol was started 2 days later (twice daily decreasing doses for 4 days, total 200 mg of Folltropin-V\(^{B}\), Bioniche AH). Cows received a CIDR\(^{B}\) device during SO to suppress a spontaneous LH surge. To collect ovarian tissue at 22 h after the LH peak by ovariectomy, a controlled LH surge was induced by a GnRH injection (1 mg of Fertagyl\(^{B}\), Intervet SP AH) at the time of CIDR\(^{B}\) removal (Vos et al. 1994 J. Reprod. Fertil. 100, 387–393). Ovaries were transported to the laboratory at 37°C directly after ovariectomy. For each animal, follicular growth was determined by counting the number of follicles >8 mm in diameter (defined as presumptive follicles). The size category of follicles was based on the volume collected after puncturing (8 to 10 mm, 10 to 12 mm, >12 mm). To determine the quality of the follicle, follicular fluid of each individual follicle was analyzed for estradiol (E2) and progesterone (P4) concentrations. Healthy follicles at 22 h after the LH peak were defined as follicles showing low E2 and high P4 (> 0.5 nmol L\(^{-1}\) (E2/P4< 1; Dielemann et al. 1983 J. Endocrinol. 97, 31–42). Statistical analysis was performed by logistic regression for grouped data (P < 0.05; mean ± SEM). The total number of follicles per animal (follicles >8 mm in diameter) did not differ between the control (18 ± 2.8) and fasted group (19.8 ± 2.9). However, the number of large follicles (>12 mm) that developed in the fasted heifers was significantly lower (2.7 ± 0.9) compared with that in the control heifers (6.0 ± 1.7). No difference was observed for the percentage of healthy follicles per animal between fasted (32 ± 9.2%) and control (56 ± 13%) heifers. These data show that follicular growth was impaired during SO treatment in fasted heifers. Although not significantly different, a numerically lower number of healthy follicles was observed in the fasted group of heifers, possibly indicating a negative influence of metabolic stress on follicular function. Whether metabolic stress affects the metabolic composition of the follicular fluid and as a consequence influences oocyte and embryo quality is under current investigation.

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176 EFFECTS OF VEHICLE AND ROUTE OF ADMINISTRATION OF LETROZOLE ON OVARIAN FUNCTION IN CATTLE


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Treatment with letrozole, a non-steroidal aromatase inhibitor, has been associated with elevated mean plasma LH concentrations, a prolonged period of dominance of the extant dominant follicle, and delayed emergence of the next follicular wave in cattle. As well, a lutetotropic effect was hypothesised by the observation that CL diameter was increased in heifers given 250 μg kg⁻¹ of letrozole divided in a 3-day regimen. The objective of the present study was to determine the effects of vehicle and route of administration of letrozole on ovarian function in sexually mature beef heifers. Ovarian function was synchronized among heifers using transvaginal ultrasound-guided follicular ablation followed by a lutetoidal dose of PGF b.i.d. 4 days later. The ovaries were subsequently examined daily by transrectal ultrasonography until ovulation was detected. On Day 3 (Day 0 = ovulation), heifers were assigned randomly to 4 treatment groups and given letrozole at a dose of 1 mg kg⁻¹ intravenously (i.v. in benzyl alcohol, n = 10) or intramuscularly (i.m. in benzyl alcohol plus canola oil 1:1 v/v, n = 10), or given a placebo (i.v. in benzyl alcohol, n = 5) or (i.m. in benzyl alcohol plus canola oil 1:1 v/v, n = 5). The ovaries were monitored daily by ultrasonography, and blood samples collected twice daily by jugular venipuncture from pre-treatment to post-treatment ovulations. Comparisons among groups were made by 1-way ANOVA for single-point measurements and by ANOVA for repeated measures for time-series data. The interovulatory interval did not differ among groups, nor did the day-to-day diameter profile of the dominant follicle of wave 1 (first follicular wave after ovulation). However, the interval between emergence of waves 1 and 2 was longer in heifers treated with letrozole i.m. (11.7 ± 0.3 days) than in controls (10 ± 0.4 and 9.5 ± 0.5 days for i.v. and i.m. controls, respectively; P < 0.05), and intermediate in heifers given letrozole i.v. (10.6 ± 0.30 days). The day-to-day diameter profile of the corpus luteum was greater (P < 0.05) and plasma progesterone concentrations tended to be greater (P < 0.06) in heifers treated i.m. with letrozole v. placebo. Plasma LH concentrations did not differ among groups, whereas plasma FSH concentrations were greater (P < 0.02) in heifers treated i.v. with letrozole v. placebo. In summary, letrozole dissolved in benzyl alcohol and given intravenously at a dose of 1 mg kg⁻¹ on Day 3 did not alter ovarian function in cattle, but the same dose given i.m. in canola oil vehicle resulted in a longer inter-wave interval, a greater CL diameter profile, and greater plasma progesterone concentrations. We conclude that i.m. letrozole in oil is a feasible route of administration and vehicle for the development of an aromatase inhibitor-based treatment for herd synchronization in cattle.

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Gene Expression

177 GENE EXPRESSION PROFILES OF IN VITRO- AND IN VIVO-DERIVED BOVINE EMBRYOS


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Microarray technology is one of the most powerful tools for gene expression profiling in animal sciences. The objectives of this study were to determine the effect of vitrification on gene expression in in vitro- and in vivo-derived bovine embryos, and to identify differential mRNA expression patterns between embryos produced by in vivo v. in vitro conditions. Three pools of in vivo- and in vitro-derived blastocyst-stage embryos were used for microarray analysis. Total RNA was isolated using the PicoPure RNA Isolation Kit (Arcturus Bioscience, Mountain View, CA). Bovine ovarian tissue total RNA was used as the reference. Total RNA samples were amplified using an Ovation® Bico WTA System (NuGEN Technologies, San Carlos, CA). The bovine 16 846-member microarrays spotted with 70-mer oligonucleotides were purchased from the Bovine Genomics Laboratory, University of Missouri. Amplified cDNA samples were labeled with Alexa Fluor 647 and 546 dyes (Molecular Probes, Eugene, OR), respectively. Combined, labeled microarrays were hybridized overnight at 42°C. Following hybridization, the slides were washed with different stringency buffers and water. After drying by centrifugation, the arrays were scanned on a GenePix 4000B scanner (Axon Instruments, Union City, CA). GenePix Pro 4.1 software was used for gridding and analysis of spot intensities. Good-quality spots were analyzed using the GeneSpring 7.3 software (Agilent Technologies, Inc., CA, Santa Clara, CA). The data were normalized per spot and per array by Lowess normalization. When comparing two treatments, the Welch t-test with Benjamini and Hochberg multiple testing correction was performed to determine the differentially expressed genes between embryo groups. Microarray experiments were performed in 3 biological and 2 technical replicates for all embryo samples. Differentially expressed genes between all embryo groups were identified. The DAVID Functional Annotation Tool was used to analyze the genes that were differentially expressed. The DAVID Functional Annotation Tool determined the co-occurrence probability and provided gene-GO term enrichment analysis. Our results were used to identify the most relevant GO terms associated with a given gene list. Differentially expressed Kyoto Encyclopaedia of Genes and Genomes pathways were as follows: Ribosome, oxidative phosphorylation, spliceosome, and oocyte meiosis were significantly upregulated in the fresh embryos, whereas sphingolipid and purine metabolism was the upregulated in the vitrified in vitro-derived embryos. Gene expression was very similar between fresh and vitrified in vivo-derived, as opposed to in vitro-derived, embryos.

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