ABSTRACTS FOR POSTER PRESENTATION

215 SUCCESSFUL OUT-OF-BREEDING SEASON ESTRUS SYNCHRONIZATION FOLLOWED BY FIXED TIME INSEMINATION IN WATERBUFFALO (*BUBALUS BUBALIS*)

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The most important barrier to the increase of buffalo productivity is an overall poor reproductive efficiency, characterized by late sexual maturity, seasonal anestrus and long periods of postpartum ovarian inactivity resulting in extended calving intervals and poor expression of estrus behavior (Singh J *et al.*, 2000, Anim. Reprod. Sci. 60–61, 593–604). Buffaloes are seasonal breeders with the highest reproductive activities during winter (short day lengths) and a high frequency of anestrus during the summer months (Singh G *et al.* 1985, Ind. J. Anim. Res. 19, 57–60). Recent research demonstrated that a combination of progesterone, estradiol benzoate and equine chorionic gonadotropin (eCG) was effective for estrus induction and synchronization in buffalo heifers under Mediterranean conditions (Barile *et al.* 2001, Livestock Prod. Sci. 68, 283–287). The aim of the present study was to investigate the impact of an estrus synchronization protocol on reproductive efficiency of water buffalo during out of the normal breeding season. A total of six heifers (21 to 23 months of age) and three cows (5, 6 and 18 years of age) were enrolled in an estrus synchronization protocol lasting for 12 days. All animals were kept under tropical conditions in the coastal part of Paraná (Antonina), about 450 km south of São Paulo. The experiment was performed in December, 2002, during the Brazilian summer season, when reproductive efficiency of buffaloes is greatly reduced. On the first day of the protocol (Day 0), animals were implanted with an intravaginal device containing 1 g of progesterone (DIB, Syntex SA, Buenos Aires, Argentina) and injected with 10 mg estradiol benzoate (Estrogin, Famavet, São Paulo, Brazil). On Day 9, the DIB implant was removed and the animals received 150 μg (i.m.) of cloprostenol (Prolise, Syntex SA, Buenos Aires, Argentina) and 2500 IU of eCG (Novormon, Syntex SA, Buenos Aires, Argentina). On Day 11, all animals received 1500 IU of hCG (Vetecor, Lab. Calier, Spain). Artificial insemination (AI) was performed on Day 12 using frozen-thawed semen from a bull of proven fertility. Only one AI was performed per heifer/cow. Pregnancies were determined by ultrasound examination at 53 days following AI and confirmed by rectal palpation at 90 days post AI. The use of this estrus synchronization protocol, followed by fixed-time insemination, resulted in four pregnant heifers (66%) and three pregnant cows (100%). Our results demonstrate that buffalo reproduction can be successful during out-of-breeding season when adequate hormonal treatment is used. Additional experiments should be done to validate the protocol.

Folliculogenesis/Oogenesis

216 TRANSVAGINAL ULTRASOUND-GUIDED BIOPSY OF THE BOVINE OVARY: A NEW TOOL FOR THE STUDY OF PREANTRAL FOLLICLES

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Current research on follicular dynamics is focussing more on primordial and preantral follicular populations. Whereas antral follicular development can be studied by ultrasound visualization, preantral follicles can be visualized only on stained ovarian tissue sections obtained through laparotomy or

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following slaughter, which does not allow dynamic follicular follow-up studies. To our knowledge, only one report has previously been published on a technique for transvaginal corpus luteum biopsy (Kot K *et al.* 1999 Theriogenology 52, 987–993). Therefore, a new method to repeatedly take ovarian biopsies in living donor cattle through ultrasound-guided transvaginal puncture of the ovarian stroma was developed and tested. Healthy cows with a normal reproductive tract upon rectal examination were prepared as for transvaginal ovum pick-up (OPU) (Bols PEJ *et al.* 1995 Theriogenology 43, 677–687). The ovary was visualized using a mechanical multiple angle 5-MHz ultrasound transducer (Pie Medical, the Netherlands), fitted next to a needle guidance system in an OPU handle. A specially designed needle carrier with a 14-gauge disposable needle (length 38 mm, outer diameter 2.1 mm, Kruuse, Japan), acting as a trocar, was inserted through the vaginal wall. A 60-cm-long disposable biopsy needle with an inner stylet having a 20-mm-long specimen notch, a cutting cannula (diameter 1.3 mm), and an automated spring-loaded handle with trigger (Quick-Core® Biopsy Needle, Cook, Denmark) was passed through the disposable needle into the ovarian stroma, under ultrasound control. Prior to insertion, the plunger of the biopsy needle was pulled back, with the needle spring-locked in the ready position. The biopsy needlepoint was positioned closely under the surface of the ovary with the stylet retracted and the specimen notch covered by the cutting cannula. With the needle in position, the stylet was advanced to expose the specimen notch within the ovarian stroma. Then, the cutting cannula was fired and a tissue sample was captured. Repeated biopsies were taken in four cows two times a week, over a two-week period, without echographically detectable detrimental effects of the reproductive tract. A tissue specimen was recovered in 25 of 30 attempts, although the dimensions of some of the biopsies were rather small. Biopsies from both the ovarian stroma and the corpus luteum (CL) were taken. They were fixed with 4% paraformaldehyde for 2 h at room temperature. Following overnight incubation in a 15% sucrose solution for cryoprotection, 17-μm cryostat sections were made and stained with hematoxylin. Study of the sections revealed small populations of primordial follicles clearly distinguishable from the surrounding ovarian stroma. Distinct groups of luteinized cells could be seen in CL biopsies. Further studies are necessary to standardize the procedure and to evaluate the effects of biopsy-taking on fertility.

217 LUTEAL REGRESSION AND FOLLICLE DEVELOPMENT FOLLOWING PROSTAGLANDIN-$F_{2\alpha}$ TREATMENT 3 DAYS AFTER OVULATION IN MARES

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The corpus luteum (CL) is responsive to exogenous prostaglandin-$F_{2\alpha}$ (PGF) 1 to 2 days after ovulation in the mare (Troedsson *et al.* 2001 Theriogenology 55, 1891–1899); however, complete and sustained demise of the CL beginning less than 5 days after ovulation in response to PGF treatment has not been documented. The present study was designed to compare the morphological and physiological response of the primary CL to PGF given at early diestrus with a more conventional treatment given at about mid-cycle. In addition, follicle status pre- and post-treatment were examined and compared between the treatment groups. On the day of pretreatment ovulation (Day 0), riding-type horse mares were randomly assigned to receive a single dose of PGF (Lutalyse, Upjohn, Kalamazoo, MI, USA; 10 mg/mare, i.m.) on Day 3 ($n = 17$) or Day 10 ($n = 17$). Beginning on either Days 3 or 10, transrectal ultrasonography was used to determine follicle and CL diameters, determine luteal tissue gray-scale scores (echogenicity), and to detect ovulation. Follicular and luteal measurements and jugular blood samples were collected daily until the post-treatment ovulation. Structural and functional regression of the CL was indicated by: 1) a progressive decrease (day effect; $P < 0.0001$) in mean diameter of the CL beginning 24 h after PGF treatment in the Day 3 and Day 10 groups; 2) a precipitous decrease ($P < 0.009$) in mean plasma progesterone concentrations within 24 h in both groups followed by a more gradual decline to basal concentrations by the second day in the Day 10 group or after the fourth day in the Day 3 group; and 3) an increase ($P < 0.02$) in mean luteal tissue echogenicity in both groups after the second day following PGF treatment. The mean intervals from PGF treatment to ovulation were not different ($P > 0.2$) between groups (combined, 9.9 days) but the mean ($\pm$ SEM) interovulatory interval was shorter ($P < 0.0001$) in the Day 3 group (13.2 ± 0.9 days; range, 7 to 20 days) than in the Day 10 group (19.2 ± 0.7 days; range, 14 to 26 days). The greater the diameter of the largest follicle at the time of PGF treatment, the shorter the interval to post-treatment ovulation in the Day 3 ($r = -0.57$, $P < 0.02$) and Day 10 ($r = -0.74$, $P < 0.001$) groups. Growth rates of the preovulatory follicles were similar ($P > 0.59$) between groups (combined, 3.6 mm/day) but the maximum diameter was smaller ($P < 0.05$) in the Day 3 group (40.5 ± 1.2 mm) compared to the Day 10 group (43.4 ± 0.8 mm). Unexpectedly, more ($P < 0.03$) double ovulations occurred in the Day 3 group (6/17, 35%) than in the Day 10 group (1/17, 6%). In conclusion, an immature CL at early diestrus responded to PGF treatment in a manner comparable to a mature CL at mid-cycle. The Day 3 group ovulated an average of 6 days earlier than the Day 10 group as a result of the difference in timing of the PGF treatment between groups. Thus, these results warrant a reassessment of the prevailing concept that the equine CL is resistant to PGF-induced regression before 5 days after ovulation, especially when considering the potential benefits of a shortened interovulatory interval and an increased double ovulation rate.

218 ULTRASOUND-GUIDED TRANSVAGINAL INJECTION OF A LOW DOSE OF FSH-LH INTO THE BOVINE OVARY AS AN ALTERNATIVE WAY TO STIMULATE FOLLICULAR GROWTH: PRELIMINARY RESULTS

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While some researchers claim a positive influence of FSH on the number of punctured follicles and retrieved oocytes in stimulated cows (Looney CR *et al.* 1994 Theriogenology 41, 67–72), others found comparable results between protocols with one stimulated v. two unstimulated ovum pick-up (OPU) sessions a week (Stubbings RB and Walton JS 1995 Theriogenology 43, 713–721). The use of FSH/LH causes explosive follicular growth and a substantial increase in ovarian blood supply when given at the superovulation dose. These drawbacks limit the repeated use of FSH stimulation

prior to OPU in a twice per week puncture protocol. The aim of this experiment was first, to investigate the feasibility of injecting FSH directly into the ovary at a substantially lower dose and, second, to study the local effects of intra-ovarian FSH injection on follicular dynamics. Six donor cows with normal reproductive tracts were prepared as for transvaginal OPU (Bols *et al.* 1995 Theriogenology 43, 677–687). The ovary was visualized using a Multiple Angle 5 MHz ultrasound transducer (Pie Medical, Maastricht, The Netherlands), fitted next to a needle guidance system in an OPU handle. A vial of freeze-dried FSH/LH (Stimufol, 500 μg porcine FSH and 100 μg porcine LH, Ulg FMV, Liège, Belgium) was restored and diluted with saline to a solution containing 10 μg pFSH and 2 μg pLH per mL. A total dose of 160 μg pFSH and 32 μg pLH, divided over 8 injections of 1 mL diluted FSH/LH in each ovary, was given to 4 animals by transvaginal ultrasound-guided injection with a 19-gauge needle. The ovaries of one negative control cow were injected with 1 mL saline using the same protocol. The sixth cow received a classical FSH stimulation as prescribed by the manufacturer by means of i.m. injections at a total dose of 500 μg FSH and 100 μg LH as a positive control. During the first session, all follicles with a diameter of >5 mm were aspirated, while prior to each injection (morning and evening), ovarian activity was checked by ultrasound examination and taped on video for all cows. Intra-ovarian FSH injection was successful since, in most cases, a small echographically dense area was seen during and immediately following injection. This area moved around following displacement of the ovary, indicating intra-ovarian disposition of the FSH. Following the four-day stimulation treatment, the average (±SD) number of follicles with a diameter >5 mm was 5.5 ± 4.2 on the ovaries of intra-ovarian injected cows, 21 in the case of the positive control cow and only 1 follicle in the negative control. These results suggest that transvaginal, ultrasound-guided injection of a low FSH/LH dose directly into the ovary might be an alternative way for ovarian stimulation prior to OPU. Additional dose-titration experiments are ongoing.

219 EFFECTS OF GROWTH HORMONE AND GnRH ANTAGONISTS ON FOLLICULAR AND OOCYTE DEVELOPMENT IN SHEEP

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Embryo output in sheep is increased when superovulatory FSH treatments are started in the presence of a high number of small follicles (2–3 mm in size) and in absence of large follicles (>6 mm, Gonzalez-Bulnes et al., 2002. Theriogenology, 57, 1263–1272). Administration of GnRH antagonists (GnRHa) suppresses large follicles (Cognie et al., 2003. Theriogenology, 59, 171–188), whereas the use of growth hormone (GH) would increase the number of small follicles (Campbell et al., 1995. J Reprod Fertil Suppl, 49, 335–350). Our aim was to evaluate the usefulness of pre-treatments with GH or GH plus GnRH antagonists for sheep embryo production. First, we studied the effects on follicular population by serial ultrasonographies. Thereafter, we determined whether such treatments can affect oocyte developmental competence. In a first trial, a total of 18 Manchega ewes were treated with intravaginal FGA sponges (Chronogest®, Intervet Int., H) during breeding season (beginning of April). Six animals received daily i.m. doses of 15 mg of ovine GH (Tuenre, GA) for 6 days, while six females received GH plus two s.c. doses of 1.5 mg of GnRHa (Antarelix™, Zentaris, G) on Days 0 and 3 of GH treatment, and six ewes acted as controls receiving saline. Number of follicles >2 mm, determined by daily transrectal ultrasonography, increased to reach significant differences on Day 4 in sheep treated with GH/GnRHa (22.7 ± 0.8 v. 16.7 ± 0.5, $P < 0.001$) and on Day 5 in ewes injected with GH (20.3 ± 0.4 v. 17.0 ± 0.6, $P < 0.05$). The second trial involved 18 Manchega ewes treated with progestagen sponges on Day 0 and distributed in three groups at the beginning of breeding season (end of July). In the first group ($n = 7$), sheep were treated with two doses of GnRHa on Days 0 and 3 after sponge insertion and with three doses of 15 mg of GH on Days 3, 4, and 5. Thereafter, ewes from this group and from a second experimental group ($n = 7$) were treated with 3 doses of 1.5 mL of FSH (Ovagen™, ICP, NZ) every 12 h, starting on the afternoon of Day 5. A third group of sheep ($n = 4$) did not receive GH/GnRHa or FSH, acting as controls. On Day 7, follicles were aspirated and the cumulus-oocyte complexes (COC) were cultured for 24 h at 38.5°C, 5% CO_2, in TCM-199 supplemented with ovine FSH (Ovagen), LH, FCS, 17-β-oestradiol, cysteamine, and sodium pyruvate (Sigma Chemical Co., MO, USA). Nuclear maturation was measured by Hoechst 33342 fluorescence. Mean number of COC was higher in GH/GnRHa + FSH group (8.7 ± 0.9 v. 6.8 ± 1.3 in FSH group, NS and 4.5 ± 0.8 in control, $P < 0.05$) due to higher number of follicles with similar recovery rates (45.0 ± 4.5, 40.3 ± 1.4, and 39.1 ± 7.1%, respectively). There were no significant differences on the ability of COC to resume meiosis, although this was higher in FSH group (63.1 ± 9.5% for GH/GnRHa + FSH, 79.5 ± 6.3% for FSH and 60.0 ± 8.8% for control group), which can indicate the necessity of a higher FSH supply to induce final development in follicles/oocytes from ewes treated with GH and GnRHa. In conclusion, the use of GH and GnRHa would help to increase the number of gonadotrophin-responsive follicles prior to gonadotrophin injections; also, adjustment of FSH treatments improved embryo yields in superovulatory protocols.

220 ENDOCRINE AND OVARIAN FUNCTION AFTER GnRH ANTAGONIST TREATMENTS IN GOATS

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In goats, as in other mammals, the use of treatment with GnRH antagonists (GnRHa) inhibits gonadotrophin secretion, causing a suppression of the growth of large ovarian follicles. Thus, GnRHa treatment could be useful to decrease the effects of dominant follicles prior to ovarian stimulation, increasing the number of gonadotrophin-responsive follicles at the start of FSH treatments and improving the ovarian response in terms of transferable embryos. However, in goats, the beneficial effects of this treatment is annulled by a high number of unfertilised ova and degenerated embryos (2003, Cognie *et al*., Theriogenology 59, 171–188), which suggests deficiencies in oocyte developmental competence per se or induced by endocrine or follicular alterations during the peri-ovulatory period. We have tested whether these failures can be related to a prolongation of gonadotrophin down-regulation and/or alterations in follicular function after cessation of the antagonist, during the period of administration of the superovulatory

treatment, around 4 days after the end of GnRHa treatment. A total of 15 does received 45-mg FGA intravaginal sponges (Chronogest®, Intervet Int, H), the first group of 10 females were treated with daily injections of 0.5 mg of the GnRHa Teverelix (Antarelix™, Zentaris, G) for 6 days from Day 5 of sponge insertion, while five does acted as controls receiving saline. Endocrine and ovarian function were monitored daily from Day −5 to Day 4 (Day 0 = day of last GnRHa injection). Pituitary activity was determined by measuring plasma FSH and LH, and follicular activity by ultrasonographic monitoring of all >2 mm follicles and by assessing plasma inhibin A levels. During GnRH antagonist treatment, the mean plasma LH concentration was lower in treated than control goats (0.5 ± 0.2 v. 0.7 ± 0.5 ng/mL, $P < 0.0005$); however, the FSH levels remained unaffected (0.8 ± 0.4 v. 0.8 ± 0.5 ng/mL). In this period, treated does also showed an increase in the number of small follicles 2–3 mm in size (10.7 ± 0.7 v. 8.4 ± 0.6, $P < 0.05$), and a decrease in both the number of follicles >4 mm in size (5.0 ± 0.3 v. 6.8 ± 0.5, $P < 0.005$) and the secretion of inhibin A (120.9 ± 10.7 v. 151.6 ± 12.6 pg/mL, $P < 0.05$). After GnRHa treatment, LH levels increased in treated goats from the day after the last Teverelix injection (Day 1), so that LH levels were the same as controls on Day 3 (0.6 ± 0.1 v. 0.6 ± 0.2 ng/mL). However, there were even greater numbers of small follicles than during the period of GnRHa treatment (15.4 ± 0.6 in treated v. 8.9 ± 0.7 in control, $P < 0.0005$). Moreover, the number of follicles >4 mm in size and the secretion of inhibin A remained lower in treated goats (3.9 ± 0.3 follicles and 84.4 ± 7.0 pg/mL v. 5.4 ± 0.5 follicles, $P < 0.05$ and 128.9 ± 14.2 pg/mL, $P < 0.05$). These results indicate that pituitary secretion of gonadotrophins is restored shortly after the end of GnRHa treatment, but the number of follicles and the secretion of inhibin A are affected. This may be relevant to the failures in ovulation and/or fertilization reported for superovulatory protocols with GnRHa pre-treatments in goats.

221 CYSTIC BOVINE FOLLICLES ARE ASSOCIATED WITH AN ALTERATION IN LOCAL GENE AND PROTEIN EXPRESSION

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Formation of persistent follicular cysts is a prevalent problem for producers of domestic agricultural animals in North America. The most common group of animals affected is dairy cattle. This condition is problematic as it renders cattle anovulatory and infertile for the duration of the cyst. Various studies have assessed the incidence of follicular cysts in dairy cattle, revealing a range of prevalence of 9–26%. Although there is information regarding the incidence of this disorder, little is known of the etiology. It is known that a complex interaction occurs between the hypothalamus, anterior pituitary, and ovary to regulate normal reproductive function. Dysregulation at any of these sites could contribute to the formation of persistent follicular cysts. The objective of this study was to determine whether local changes in gene and protein expression are present in cystic follicles. Transvaginal aspirations from follicles that had been greater than 2.5-cm diameter for at least 10 days were collected. Aspirates were centrifuged, and granulosa cells and follicular fluid were separated. Granulosa cells were lysed, and RNA and protein was isolated. For immunohistochemistry, bovine slaughterhouse ovaries with follicles greater than 2.5 cm in diameter were dissected, fixed, and processed. Western blot analysis and RT-PCR were performed on protein and RNA samples, respectively. Cystic and control follicles were analyzed for expression of vascular endothelial growth factor (VEGF) and its receptor VEGF-R2 and members of the insulin-like growth factor (IGF) family, which are known to mediate a host of cellular events during follicular development. Cystic follicles exhibited a significant increase in VEGF and IGF-I protein concentrations and a reduction in VEGF-R2 and the type 1 IGF receptor. Immunohistochemical analysis demonstrated increased ligand staining and reduced receptor expression in granulosa cells of cystic follicles. These results indicate that there is an altered growth factor profile in cystic follicles and suggest that intra- as well as extra-follicular dysregulation is important in the etiology of this reproductive disorder.

222 THE ROLE OF TUMOR NECROSIS FACTOR-RELATED APOPTOSIS INDUCING LIGAND DURING FOLLICULAR ATRESIA IN PIG OVARIES

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Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) can induce cell death by binding to its receptors (DR4 and DR5). However, binding to DcR1 or DcR2 cannot induce apoptosis. DcRs compete with DRs. TRAIL has been reported to induce apoptosis in various tumor cells but not in normal cells. However, a recent study revealed that TRAIL induces apoptosis in normal hepatocytes of human but not in those of rat, mouse, or rhesus monkey, indicating that there are species-specific differences in TRAIL and receptor systems. In the present study, we demonstrated Immunohistochemical, Western immunoblotting, and reverse transcription-polymerase chain reaction analyses (RT-PCR) of TRAIL and DR4 in granulosa cells during follicular atresia in pig ovaries. For immunohistochemistry, pig ovaries obtained at a local slaughterhouse were fixed with 20% buffered formalin. For Western blotting and RT-PCR analysis, individual preovulatory antral follicles were dissected from the ovaries. Based on morphological and endocrinological criteria, the antral follicles were divided into three categories as follows: healthy, early stage of atresia, progressed stage of atresia. Significant increases were demonstrated in TRAIL protein and mRNA levels during atresia, but not in DR4 protein. Moreover in an in vitro apoptosis-inducing assay using cultured granulosa cells prepared from healthy follicles, we showed that more than 200 ng/mL TRAIL could activate caspase-3 and induce apoptotic cell death in a dose- and time-dependent manner, but less than 100 ng/mL of TRAIL could not induce apoptosis. When DcR1 was removed from the cell membrane of granulosa cells, a lower dose of TRAIL could induce apoptosis. The present findings suggested that the TRAIL can induce granulosa cell apoptosis, and that DcR1 blocks TRAIL-induced apoptosis in granulosa cells of healthy follicles in porcine ovaries.

223 DEVELOPMENTAL PATTERN OF SMALL (1–3 mm) ANTRAL FOLLICLES IN THE BOVINE OVARY

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Knowledge about the developmental pattern of small follicles (<4 mm) will be crucial to understanding the endogenous control of folliculogenesis and to developing methods to control it for clinical purposes. This study was designed to characterize the developmental pattern of 1–3 mm follicles and to determine, retrospectively, the stage at which the future dominant follicle first attains a size advantage among follicles in the cohort. In Experiment 1, the ovaries of Hereford-cross heifers ($n = 18$) were examined daily by high resolution transrectal ultrasonography for one natural interovulatory interval to evaluate changes in the number of 1–3-mm follicles in relation to the wave pattern of follicles ≥4 mm. Interovulatory interval was divided into 2- and 3-wave based on number of waves exhibited by heifers. In Experiment 2, the ovaries of Hereford-cross cows ($n = 9$) were examined every 6 h from Day 5 to Day 13 (Day 0 = ovulation) to monitor precisely the diameter changes of individual follicles ≥1 mm during emergence of the second follicular wave. Data were analyzed by Proc. Mixed procedure for repeated measures (Littell RC *et al.*, 2000 Stat in med 19, 1793–1819) in the Statistical Analysis System software package (SAS version 8.2 for MS Windows; 2002 SAS Institute Inc. Cary, NC, USA). Results of Experiment 1 revealed a day effect ($P < 0.05$) on the number of small (1–3 mm) follicles, with a peak ($P < 0.05$) 1 or 2 days before wave emergence (defined as the day the dominant follicle was first detected at 4 mm), followed 3 to 4 days later by a peak ($P < 0.05$) in the number of large follicles (≥4 mm). There was an inverse relationship between the number of small and large follicles during Wave 1 ($r = -0.66$; $P = 0.05$) and Wave 2 ($r = -0.62$; $P = 0.04$) in 2-wave interovulatory intervals. Similarly, an inverse relation was detected between the number of small and large follicles for Wave 1 ($r = -0.79$; $P = 0.01$) and Wave 3 ($r = -0.90$; $P < 0.01$) but not for Wave 2 ($r = -0.57$; $P = 0.14$) in 3-wave interovulatory intervals. The number of 1–3-mm follicles detected in anovulatory waves did not differ ($P = 0.53$) between 2- versus 3-wave interovulatory intervals; however, a difference ($P < 0.05$) was observed between anovulatory and ovulatory waves in 3-wave interovulatory intervals but not ($P = 0.63$) in 2-wave interovulatory intervals. Experiment 2 permitted the identification of the future dominant follicle at a diameter of 1 mm and its emergence at 6–12 h earlier than the largest subordinate follicle ($P < 0.01$). Emergence of the future dominant ($r = 0.71$; $P = 0.05$) and 1st subordinate ($r = 0.78$; $P = 0.02$) follicles was temporally associated with a wave-eliciting rise in circulating concentrations of FSH. Growth rate of the dominant and the 1st subordinate follicle differed ($P < 0.01$) from 2nd subordinate follicle at 84 h after their detection at 1 mm. It was concluded that small antral follicles (1–3 mm) develop in a wave-like manner, and apparent selection of the dominant follicle was manifest much earlier than previously documented.

224 FOLLICULAR GROWTH IN SHEEP SUPEROVULATED WITH FSH AFTER PRE-TREATMENT WITH GnRH ANTAGONISTS

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In sheep, the injection of a single dose of 1.5 mg of the GnRH antagonist Teverelix (Antarelix™, Zentaris, Frankfort, Germany) eliminates large dominant follicles and increases the number of smaller follicles (2–3 mm in size) in a short period of time (Lopez-Alonso *et al.*, 2003. Reprod. Abstr. Ser., 30:71). This treatment would be beneficial for increasing the efficiency of ovarian stimulatory protocols, since embryo output is enhanced in the presence of a high number of small follicles, coincidentally with the absence of large follicles, at starting the FSH treatment. However, possible effects of this single high dose of GnRH antagonist on the capacity of follicles to develop in response to FSH treatments has not been determined. In this study, we have characterized patterns of follicular development during a superovulatory treatment with purified ovine FSH (Ovagen™, ICPbio, Auckland, NZ) in sheep treated i.m. with 1.5 mg of Antarelix ($n = 6$) or saline ($n = 4$) on Day 9 after the insertion of a progestagen sponge (Chronogest®, Intervet Int, Boxmeer, The Netherlands). Ewes were superovulated with eight decreasing doses (1.5 × 3, 1.25 × 2 and 1 × 3 mL) of Ovagen™ injected twice daily from Day 12 of sponge insertion. Number and size of all >2-mm follicles was determined by transrectal ultrasonography just prior to every FSH injection from the first dose to the withdrawal of progestagen sponges. At the start of the gonadotrophin treatment on Day 12, ewes treated with GnRH antagonist showed a higher number of 2–3 mm follicles than control ewes (16.2 ± 3.8 v. 5.3 ± 0.3, $P < 0.05$), and a lower number of >4 mm follicles (2.2 ± 0.5 v. 5.0 ± 0.6, $P < 0.01$). Thereafter, administration of Ovagen™ induced a significant rise in the number of >4 mm follicles at sponge removal in both groups ($P < 0.0005$ for treated ewes and $P < 0.01$ for controls). This number was higher in females treated with GnRH antagonist than in control sheep, although differences did not reach statistical significance (19.3 ± 3.8 v. 12.7 ± 5.4). Current data confirm that administration of a single dose of GnRH antagonist decreases presence of large follicles and increases the number of smaller follicles at the first day of FSH injection. This pre-treatment does not affect competence of follicles to grow in response to superovulatory FSH treatments. Moreover, the number of preovulatory follicles at sponge removal was higher than in untreated ewes. We conclude that the pre-treatment with a single dose of 1.5 mg of Antarelix™ on Day 9 of sponge insertion, three days before starting the FSH treatment in a superovulatory protocol, might increase the ovarian response and, thereafter, the yields of follicles.

225 GROWTH OF OOCYTES IN PIG PRIMORDIAL FOLLICLES XENOTRANSPLANTED INTO SCID MICE

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The mammalian ovary is endowed with a large number of primordial follicles that contain small oocytes. A limited number of these oocytes initiate growth, whereas others are either degenerate or remain as completely resting oocytes throughout the reproductive life of the female. The mechanism for the initiation of oocyte growth is not understood well. Small oocytes in primordial follicles of newborn rodents start to grow in

the cultured ovary or ovarian tissue. For domestic animals, however, culture systems for mouse oocytes have not been valid. Xenotransplantation of ovarian tissues to immunodeficient mice can be a substitute for an effective culture system for small oocytes. Indeed, recent reports reveal the growth of small oocytes of fetal and newborn animals in xenografts (Hosoe *et al.*, 2001; Kaneko *et al.*, 2003 Biol. Reprod. 53, 931–939). This experiment was conducted to study the growth of oocytes in primordial follicles of adult pig (6-month-old) in comparison to those of newborn pigs (10-day-old) in xenografts. The effect of the sex of host mice on oocyte growth in xenografts was also examined. Cortical slices containing only primordial follicles were collected under a dissection microscope from the ovaries of 6-month-old gilts ($n = 8$) and 10-day-old piglets ($n = 6$). Size of the slices was about 2 mm × 1 mm × 0.5 mm. Each slice was cut into 2 pieces; one was fixed for histological examination and the other was transplanted. For transplantation, 6- to 8-week-old male and female SCID (severe combined immune deficiency) mice were anesthetized, their left kidneys were exteriorized, and cortical slices were inserted under the kidney capsules. After 2 months, the grafts were recovered and processed for histological examination. Histological examination confirmed that the cortical slices contained only primordial follicles before transplantation. After transplantation, 47% (171/364) of the primordial follicles of adult pig ovaries survived in the xenografts but none of those developed into primary follicles or beyond. The mean diameter of the oocytes after transplantation was 32.1 ± 0.7 μm ($n = 171$) which was similar to that of the oocytes before transplantation (30.7 ± 0.8 μm, $n = 364$). On the other hand, in the xenografts of newborn pig ovaries, 13.5, 9.7 and 0.3% of 1122 follicles developed to the primary, secondary and antral stages, respectively, in male SCID mice. In the female SCID mice, there were no antral follicles but the distribution of primordial, primary and secondary follicles was 84.5, 9.4 and 6.1%, respectively, among a total of 1094 follicles. The mean diameter of the secondary follicles in the xenografts in the male SCID mice was 263.3 ± 92.0 μm ($n = 109$) which was significantly higher than that of the secondary follicles in the female SCID mice (189.3 ± 44.2 μm, $n = 98$) ($P < 0.05$, *t*-test). The results show that primordial follicles of 6-month-old pig ovaries survive but do not develop in xenografts, whereas newborn pig primordial follicles develop to the antral stage. This suggests that the growth property of the oocytes in primordial follicles in adult pig ovaries is different from that in newborn pigs.

226 HEAT SHOCK OF GERMINAL VESICLE-STAGE BOVINE OOCYTES REDUCES EMBRYO DEVELOPMENT

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Culture of germinal vesicle (GV)-stage oocytes at an elevated temperature occurring in heat-stressed dairy cattle reduced ability of oocytes to progress to metaphase II after resumption of meiosis (Payton RR *et al*., 2003 Biol. Reprod. 68, 343 abst). The objective of this study was to evaluate embryo development of oocytes heat-shocked at GV stage. To prevent cumulus-oocyte complexes from resuming meiosis after removal from follicles, oocytes were cultured in roscovitine (cell cycle inhibitor of $p34^{cdc2}$/cyclin B kinase; 50 μM) for 24 h (McCann LM *et al*., 2000 Biol. Reprod. 64, 141 abst and Payton RR *et al*., 2003 Biol. Reprod. 68, 343 abst) showed that roscovitine is effective for maintaining >90% of oocytes at GV-stage in a reversible manner. Germinal vesicle-stage oocytes were cultured at 38.5°C for 24 h (experimental control) or 41.0°C as follows: HS 0–6 (41°C for 6 h, 38.5°C for 18 h), HS 0–12 (41°C for 12 h, 38.5°C for 12 h), HS 12–24 (38.5°C for 12 h, 41°C for 12 h), HS 18–24 (38.5°C for 18 h, 41°C for 6 h), or HS 0–24 (41°C for 24 h) in 5.5% CO_2 and humidified air. In addition, a group of COC were not cultured in roscovitine but placed in maturation medium (lab control). After a total of 24 h, COC were washed extensively of roscovitine and cultured for an additional 24 h in maturation medium. Oocytes presumed mature were fertilized with Percoll-prepared frozen-thawed semen. Presumptive zygotes were cultured in KSOM containing 1X nonessential amino acids in 5.5% CO_2, 7% O_2, and 87.5% N_2 at 38.5°C in humidified air. Cleavage and development to blastocyst were recorded on Days 3 and 8 post-insemination, respectively. Data were collected in 7 replicates and analyzed as an incomplete block using mixed models of SAS (2000) after testing for normality. Use of roscovitine for maintaining oocytes at GV-stage for 24 h did not alter cleavage (80.5 and 73.4%; SEM = 5.8; lab and experimental controls), development to 8–16 cell (50.4 and 52.6%; SEM = 4.6; lab and experimental controls), or blastocyst (29.7 and 24.8%; SEM = 3.2; lab and experimental controls) stages. Culture of GV-stage oocytes at 41°C for up to 24 h did not increase lysis (8.0–11.1%; SEM = 2.7). Heat shock of GV-stage oocytes for as few as 6 h reduced the proportion developing to 8–16 cell stage after release from inhibitor (Table 1). When experimental control and HS 0–6 were pooled for comparison to HS 0–12, effects of heat shock for reducing development to blastocyst were noted ($P < 0.005$). Moreover, negative effects of heat shock for reducing developmental competence of GV-stage oocytes increased as duration of heat shock increased (linear contrast; experimental control, HS 0–12, and HS 0–24; $P < 0.04$). Results indicate that a physiologically relevant elevated temperature for as few as 6 h compromises continued development of GV-stage oocytes. Seasonal depressions in fertility of heat-stressed cattle may be due in part to direct effects of elevated temperature on GV-stage oocytes.

Table 1.

Treatments	No.	Cleavage (%)	4-cell (%)	8–16-cell (%)	Blastocysts (%)
Expt. Control	179	71.8	9.3^{c}	52.6^{a}	20.1^{ab}
HS 0–6	260	69.5	18.6^{b}	38.9^{b}	23.3^{a}
HS 0–12	250	65.4	22.9^{ab}	28.4^{bc}	13.0^{bc}
HS 12–24	252	69.1	22.2^{ab}	34.0^{b}	18.1^{ab}
HS 18–24	216	70.9	21.0^{ab}	38.8^{ab}	19.7^{a}
HS 0–24	265	62.3	25.9^{a}	19.4^{c}	11.8^{c}
SEM		5.1	3.0	4.3	2.7
P-value		0.67	0.006	0.0008	0.008

^{a,b,c}Values with different superscripts in the same column differ significantly.

227 OVARIAN FOLLICULAR DYNAMICS IN POSTPARTUM MEDITERRANEAN ITALIAN BUFFALOES

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In cattle and buffaloes resumption of ovarian activity, as expressed by first post partum ovulation and follicle turnover leading to periodic estrus cycles and ovulations, can be affected by many factors among which uterine infections and dietary supplementation play a leading role. In addition, buffaloes are considered seasonal animals characterized by a tendency to reproduce in correspondence to a decrease of day length. This aspect may further affect their reproductive efficiency by delaying or interrupting ovarian activity if calving occurs in transitional (i.e., from end winter into beginning of spring) or unfavourable (spring-summer) periods of the year, when an increase of daylight hours occurs. The aim of this study, carried out in the months of February to May, was to understand the effect of increasing daylight hours over resumption of postpartum ovarian follicular dynamics and estrus cycles. Mixed parity Mediterranean Italian buffaloes ($n = 20$) were monitored daily by ultrasonography from calving until first postpartum ovulation and following cycle or until 60 days if no ovulations had been recorded. Ultrasound monitoring was performed by using a 7.5 MHz linear-array probe and an Aloka SSD-500 monitor. No pharmacological intervention was allowed during the course of the study and animals were kept under similar feeding and farm management conditions. All animals exhibited a mild to moderate uterine inflammation ($n = 13$; 65%) or aspecific infection ($n = 7$; 35%) after calving. Infections were manifested as purulent discharge and as ultrasound detection of highly echogenic debries in the uterine lumen. Only one animal retained pathological accumulation in both horns for the entire duration of the study. Among the 20 buffaloes under study, 12 (60%) exhibited postpartum ovulations, ranging from 9 to 45 days. In eight buffaloes (40%), such ovulations occurred in the ovary contralateral to the ovary bearing the gravidic CL. Following postpartum ovulations, seven buffaloes (35%) underwent a complete cycle leading to ovulation. Three of such cycles (15%) culminated with a second ovulation in the same ovary where post partum ovulations had occurred. Among the full cycles following postpartum ovulations, four of them were one-wave, with a range of 8 to 19 days, whereas the remaining three cycles were two-wave with a range of 17 to 22 days. Eight buffaloes (40%) were reported in anovulatory condition following parturition, although in four of them both ovaries displayed a typical follicle turnover. In conclusion, resumption of ovarian activity and reproductive efficiency in water buffaloes can be affected by increasing day length as evidenced by a high rate of postpartum anovulatory conditions and reduced follicle turnover.

228 DOMINANT FOLLICLE DEVIATION IN GUZERA BREED (*BOS INDICUS*) COWS

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Follicular dynamics of Zebu Cattle are characterized by lower maximum diameter and persistence of dominant follicles, when compared to European breeds. However, the main effects of follicular dominance on subordinated follicle population, and consequently on oocyte recovery and embryo production, have been shown to be similar among breeds. The aim of this study was to evaluate the dominant follicle establishment period, indicated by follicle divergence, in Zebu cows. Pluriparous, non-lactating Guzera breed (*Bos indicus*) cows ($n = 5$), presenting a mean body weight of 518.0 ± 48.5 kg and similar body score condition (ranging from 3.5 to 4, in a 1 to 5 scale), were used. In a first trial, follicular dynamics were evaluated daily during 1 ($n = 2$) or 2 ($n = 3$) estrous cycles, using a portable ultrasound device equipped with a linear rectal 5 MHz transducer (Aloka SSD500, Aloka Co., Tokyo, Japan). In a second trial, estrous cycles were synchronized using progesterone intravaginal-releasing devices (CIDR, Pharmacia, Sao Paulo, Brazil), and follicular dynamics were evaluated every 6 h after ovulation, during the first follicular wave, until the largest follicle achieved a diameter of 10 mm. During each evaluation ovaries were mapped, and evolution of follicles followed individually. Data are normalized for the moment of follicular divergence, and analyzed by ANOVA. Results are presented as mean ± SD. Data from the first evaluations confirmed the occurrence of cycles with three (4/8) and four (1/8) follicular waves, despite a mean cycle length of 19.1 ± 1.9 days, with a maximum diameter of 11.6 ± 2.4 mm for non-ovulatory follicles, and 14.4 ± 0.5 mm for ovulatory follicles. Follicular deviation during the first follicular wave occurred 49.2 ± 5.0 h after ovulation, when the largest follicle presented a diameter of 7.3 ± 0.6 mm, and the second largest follicle 6.8 ± 0.7 mm. Growth rates of the dominant and the largest subordinated follicles were similar before (0.35 mm/6 h vs. 0.33 mm/6 h, respectively; $P > 0.05$), but significantly different after deviation (0.52 mm 6 h v. −0.04 mm 6 h; $P < 0.05$). These results shows that the lower maximum diameter and persistence of dominant follicles, in Zebu cattle, is partially compensated by an earlier dominant follicle deviation.

229 CHARACTERIZATION OF CONNEXIN 43 IN THE CANINE OVARIAN FOLLICLE

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Connexin 43 (Cx43) is the most abundant gap-junction protein in ovarian follicle cell interactions and has been well characterized for several mammalian species. However, the involvement of Cx43 in canine follicular development has not been extensively investigated. The objective of this study was to 1) characterize the spatial patterns of Cx43 localization within the canine ovarian follicle at various physiological states and during the estrous cycle (prepubertal, anestrus, proestrus, estrus and diestrus), and 2) to characterize the canine Cx43 mRNA transcript. The spatial expression pattern of Cx43 protein was evaluated by immunofluorescence microscopy in canine ovaries. Cx43 was not detected in primordial follicles, but was detected in primary and secondary follicles at each physiological state at all granulosa cell borders. In secondary follicles, definitive, punctate staining

patterns were localized along mural granulosa cells and also in the surrounding granulosa-cumulus cell borders. Notably, more intense staining was observed in the corona radiata cells immediately surrounding the oocyte, as well as in trans-zonal projections and at the perivitelline membrane. Patterns of localization were most similar between proestrus and diestrus, and between prepubertal and anestrus in secondary follicles. Estrus-stage follicles showed a decrease in localization at the corona-oocyte cell borders as compared to proestrus and diestrus. In large, healthy antral follicles from proestrus and estrus stages, Cx43 was present in the stroma, theca, and granulosa layers. However, antral follicles from estrus-stage ovaries showed more intense staining in the mural granulosa and theca layers, and less intense in the stroma as compared to those of proestrus stage. The most intense pattern of punctate staining was observed in the corpora lutea of diestrus-stage ovaries. Additionally, gene-specific primers were designed from highly conserved regions of Cx43 mRNA among bovine, human and mouse. RNA was isolated from canine uterus and used as a template for RT-PCR. The PCR products were then sequenced and verified in GenBank to assess homology. The sequenced coding region for canine Cx43 mRNA shares 93% sequence homology with bovine vascular smooth muscle. Canine specific primers for this sequence have been designed, and expression analysis studies in canine ovarian follicles are currently underway. These results indicate that the pattern of localization of Cx43 is similar to that reported for the cow and the pig, except that in canine ovarian preantral follicles, Cx43 is also localized to the peri vitelline membrane. Additionally, these results suggest that the localization of Cx43 is dependent on the physiological state of the ovary, and is likely necessary for folliculogenesis and subsequent oocyte development in canines.