RESUMO.- [Efeito do acetato de medroxi-progesterona sobre o crescimento filicular e expressão endometrial de ciclooxigenase-2 (COX-2) durante o ciclo estral de bovinos.] Este estudo teve como objetivo avaliar o efeito do acetato de medroxi-progesterona (MAP) com ou sem benzoato de estradiol (BE) sobre o crescimento folicular durante o ciclo estral bovino. No primeiro experimento, vacas da raça Hereford foram sincronizadas com um análogo sintético de PGF2α e tratadas com duas doses de MAP (250 ou 500 mg) com ou sem EB para 7 dias starting on day 8 of the estrous cycle. Follicular growth was inhibited (P<0.05) in all cows except controls and those receiving 250mg MAP without EB. Seventy-five percent of the animals (15/20) showed estrus on days 21 and 22 of the cycle rather than at MAP withdrawal, demonstrating that these treatments did not induce estrus. To determine whether the EB treatment altered endometrial sensitivity to oxytocin and thus the luteolytic cascade, multiparous pre-synchronized cows received 5 mg of EB followed 6 hours later with 5 IU of oxytocin (OT; n=9). Eight hours after EB injection, endometrial fragments were collected from the cows on days 4, 13 and 17 of the estrous cycle and COX-2 gene expression was measured by PCR. EB increased COX-2 mRNA levels only on day 17 of the estrous cycle (P<0.05). In conclusion, MAP alone or associated with EB is able to suppress bovine follicular growth. However, EB in the presence of MAP is not efficient to induce luteolysis in cows when injected on day 8 of the estrous cycle.

INDEX TERMS: Follicular growth, estradiol benzoate, medroxy-progesterone acetate, beef cattle, luteolysis.

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pré-sincronizadas receberam 5mg de BE, seguidos, após 6 horas, de 50 UI de ocitocina (OT; n=9). Oito horas após a administração de BE, colheram-se fragmentos endométriais das vacas, nos dias 4, 13 e 17 do ciclo estral, mensurando-se a expressão gênica de COX-2 através de PCR. O BE aumentou os níveis de RNAm de COX-2 apenas no dia 17 do ciclo estral (P<0,05). Em conclusão, o MAP isolado ou associado a BE é capaz de suprimir o crescimento folicular bovino. Entretanto, o BE, na presença de MAP é ineficaz na indução da luteólise bovina, quando injetado no oitavo dia do ciclo estral.

TERMOS DE INDEXAÇÃO: Crescimento folicular, benzoato de estradiol, acetato de medroxi-progesterona, bovinos de corte, luteólise.

INTRODUCTION

Although the use of progesterone to inhibit ovulation and synchronize estrus has been widely employed in cattle (Martinez et al. 2007, Small et al. 2009), the use of synthetic progestins in cattle is less well known. Medroxy-progesterone acetate (MAP) is cheaper than natural progesterone and is a potentially good option for extensive farming practices in Brazil. MAP has been used to induce estrus in post-partum beef cattle (Medeiros Bastos et al. 2004, Terra et al. 2008), and its use in synchronization of cyclic cattle within an Ovsynch protocol has been reported (Cavestany et al. 2003, Terra et al. 2008). Depending on stage of cycle, the dominant follicle subject to exposure to MAP may be the follicle that ovulates at MAP withdrawal (Burke et al. 1996), therefore the impact of MAP synchronization protocols on follicle dynamics requires elucidation.

High concentrations of progesterone have been shown to limit follicular growth (Adams et al. 1992, Burke et al. 1996) and induce atresia of persistent follicles (Savio et al. 1993, Stock & Fortune 1993, Anderson & Day 1994). Additionally, exogenous estradiol induces atresia of the dominant follicle (Bo et al. 1995, Kinder et al. 1996) and induces luteolysis (Thatcher et al. 1986), leading to the emergence of a new follicle wave 3 to 4 days after the initiation of treatment (Kinder et al. 1996). Although the feedback mechanisms of estradiol and progesterone on LH secretion are different (Price & Webb 1988), when administered in combination, estradiol greatly enhances the ability of progesterone to suppress the LH surge in ovariectomized ewes and cows (Stumpf et al. 1993, Cupp et al. 1995). The effect of exogenous estradiol on MAP synchronization protocols is not known.

The aim of this experiment was to determine the influence of two different concentrations of MAP, with or without exogenous estradiol, on follicular growth and induction of estrus under typical extensive farming conditions in Brazil. As the expression of estrus is dependent on the ability of treatments to induce luteolysis, we also measured the effect of exogenous estradiol on uterine sensitivity to oxytocin.

MATERIALS AND METHODS

Experiment 1

Twenty Hereford cows at a body condition score of 4 (1 = lean; 5 = overweight) were housed at the Embrapa Sul experimental farm in Bagé, Rio Grande do Sul. The animals were pre-synchronized by an injection of 500μg of cloprostenol (Sincrocio, Ourofino, Brazil). Twenty-four hours later, the animals were observed for detection of estrus.

After the detection of estrus, the animals were randomly allocated to one of 5 groups as follows: control group (n=3); group MAP250BE (n=4) received an intra-vaginal device (IVD) with 250mg MAP (Purifarma, SP, Brazil) and an intramuscular (IM) injection of 5 mg of estradiol benzoate (EB) (Purifarma, SP, Brazil) diluted in vegetable oil; group MAP250 (n=5) received an IVD containing 250mg MAP; group MAP500BE (n=4) received an IVD containing 500mg MAP and an IM injection of 5mg of EB diluted in vegetable oil; and group MAP500 (n=4) received an IVD containing 500mg MAP. The treatments were given on day 8 of the estrous cycle. The IVDs were removed on the day 14 of estrous cycle, and estrus detection started thereafter.

All groups were evaluated by ultrasound through a Pie Medical model 480 (Pie Medical, The Netherlands) equipped with a transrectal linear 5 MHz real time transducer, and connected to a VHS recorder set. During each evaluation, a complete scanning of the ovaries was performed, and the diameters of the largest and second largest follicles were measured. The evaluations took place on the first day of the treatment and once every 2 days for 14 days.

Experiment 2

Animals and sample collection. Eighteen Red Angus and Nelore-cross cows, between three and four years old, were raised extensively in a private farm in Sao Gabriel, Rio Grande do Sul, Brazil. Every animal was pre-synchronized with 500μg cloprostenol. The animals were separated in two groups. The treated animals (n=9) received 5mg EB and followed six hours later with 50 IU oxytocin (OT) (Purifarma, SP, Brazil). The control group (n=9) received 50 IU OT only. The treatments were applied at three different moments of estrous cycle, day 4, 13 or 17; thus there were three animals per group/time (2 Angus and 1 Nelore). The OT challenge was used because estradiol upregulates OT receptor mediated expression of COX-2 (Burke et al. 1996). Two hours after OT injection, fragments of endometrium were collected under sedation and caudal epidural anesthesia. An incision was performed on the dorsal region of the vagina, vertically and over the first portion of the cervix. The tissues were stored in RNA Later ® (Qiagen) and frozen in liquid nitrogen. All animal procedures were approved by the Bioethics Committee of the College of Veterinary Medicine, Federal University of Santa Maria.

Nucleic acid extraction & semi-quantitative RT-PCR.

Gene expression was assayed by RT-PCR. Total RNA (1 mg) was first treated with 1 U DNase (Promega, Madison, WI) at 37°C for 30 min to digest any contaminating DNA, followed by adding 1ml EDTA stop buffer at 65°C for 10 min. The RNA was reverse transcribed in the presence of 1mM oligo(dT) primer and 4 U Omniscript RTase (Qiagen, USA), 0.25mM dideoxynucleotide triphosphate (dNTP) mix, and 19.33 U RNase Inhibitor (Amersham Biosciences, USA) in a volume of 20ml at 37°C for 1 h. The reaction was terminated by incubation at 93°C for 5min.
Bovine-specific primers for COX-2 (Pfaffl et al. 2003) and histone H2a (Hayashi et al. 2003) were used. PCR of fixed amounts of RNA indicated that H2a amplicon abundance was stable across treatments, whereas amounts of another housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase, were found to vary. An aliquot (1ml) of the cDNA template was amplified by PCR using 0.25ml (2.5 U) Taq Polymerase (Amersham Pharmacia Biotech Inc., USA) in a 20-ml PCR buffer (Amersham Pharmacia Biotech Inc.) containing 0.1 mM dNTP mix, and 0.2 mM specific primers. After an initial denaturation step for 3min at 94°C, target cDNA was amplified with a denaturation step at 94°C for 60 sec, annealing for 45sec at 60°C (COX-2) or at 58°C for 30sec (H2a), and elongation at 72°C for 1min. All reactions were terminated with a final elongation at 72°C for 5min. Reactions was performed for 30 cycles for COX-2 and for H2a, 29 cycles.

The PCR products were separated on 2% agarose gels containing 0.001% ethidium bromide, and visualized under UV light. Quantification of band intensity was performed with Image J (NIH, USA). The use of animals in these experiments was in accordance with the Guide to the Care and Use of Experimental Animals (Canadian Council on Animal Care, 315-350 Albert Street, Ottawa, Ontario, K1R 1B1) and was approved by the animal care committee of the authors’ institution.

Statistical analysis
The effect of treatments on diameter of the largest follicle was tested by ANOVA. Dominance was determined by measuring the diameters of the two largest follicles by ultrasound. ANOVA for repeated measures was used to compare results in the same group, where the class was the day and the factor of repetition was the cow.

Data that did not follow a normal distribution pattern (Shapiro-Wilk’s test) were transformed to logarithms. Homogeneity of variance was tested with O’Brien and Brown-Forsythe’s tests. All analytical procedures were performed with JMP software (SAS Institute, USA) with treatment group as main effect and cow replicate as a random variable in F-test. For gene expression data, housekeeping gene abundance was included as a covariate in the main effects model. Differences between means were tested with the Tukey-Kramer’s HSD test. Data are presented as means ± SEM.

RESULTS
Experiment 1. Wave dynamics of the dominant and subordinate follicles in each treatment group are shown in Figure 1. The growth of the dominant follicle was suppressed in animals receiving 500mg MAP.
MAP with or without EB, and in animals receiving 250mg MAP alone, in which 300mg MAP failed to alter the growth of the dominant follicle (Cavestany et al. 2003), or where rising concentrations of the synthetic progestin norgestomet inhibited growth of the dominant follicle (Sanchez et al. 1995). It is known that estradiol enhances the inhibitory influence of progestagens on LH release (Price & Webb 1988). In agreement with this, the addition of EB to the 250mg MAP dose inhibited follicle growth compared to MAP alone. Although LH pulse secretion was not measured, it is likely that EB enhanced the inhibitory effect of progesterone on LH secretion. Despite the inhibition of follicle growth during MAP+EB treatment, data suggest that follicle growth following IVD removal is not affected by pretreatment with MAP (Borges et al. 2008).

In the present experiment, estrus was not synchronized but occurred at the normal time, on day 21 and 22 of the cycle. This was because the animals were pre-synchronized with PG and all treatments started on day 8 of the cycle. All animals thus had a functional corpus luteum during and after IVD placement. This design allowed the study of follicle dynamics under controlled conditions, and allowed us to determine whether EB would cause luteolysis in this model. As the follicle data and lack of estrus synchronization shows, EB did not induce luteolysis when given with MAP on day 8 of the cycle.

Estradiol is believed to induce luteolysis by increasing endometrial OT receptor expression, and thus OT induction of COX-2 and PG synthesis (Thatcher et al. 1986, Kombe et al. 2003). To determine whether the inability of EB treatment to induce estrus in this study was related to responsiveness to OT, COX-2 mRNA levels were assessed following OT challenge. EB did not increase COX-2 expression until day 4 of the cycle, consistent with the lack of luteolysis and synchronization of estrus observed in Experiment 1. This is later than the mid-cycle response reported earlier (Thatcher et al. 1986), and may be influenced by dose of EB or OT used in the present study.

In conclusion higher doses of MAP alone or lower doses of MAP in conjunction with EB are able to suppress growth of the dominant follicle in cattle. Although the injection of effects of MAP on follicle dynamics, and whether exogenous estradiol was capable of inducing luteolysis and a new follicle wave. This information has implications for implanting simple and cost effective estrus synchronization protocols suitable for extensive farming practices in Brazil.

Altering progesterone concentrations during the cycle has impact on gonadotropin secretion. High levels inhibit LH secretion whereas lower sub-luteal levels result in raised LH secretion (Stock & Fortune 1993). Extended periods under sub-luteal concentrations of progesterone, such as CIDR or MAP synchronization protocols, prolong the period of follicular dominance, maintain high levels of estradiol, and reduce oocyte viability and fertility (Stock & Fortune 1993, Mihm et al. 1994). In Experiment 1, we demonstrated that MAP at a dose of 250 mg alone is incapable of blocking follicular growth, whereas at the higher dose of MAP, the dominant follicle failed to reach its normal size. These data agree with earlier studies, in which 300mg MAP failed to alter the growth of the dominant follicle (Cavestany et al. 2003), or where rising concentrations of the synthetic progestin norgestomet inhibited growth of the dominant follicle (Sanchez et al. 1995).

It is known that estradiol enhances the inhibitory influence of progestagens on LH release (Price & Webb 1988). In agreement with this, the addition of EB to the 250mg MAP dose inhibited follicle growth compared to MAP alone. Although LH pulse secretion was not measured, it is likely that EB enhanced the inhibitory effect of progesterone on LH pulse frequency. Despite the inhibition of follicle growth during MAP+EB treatment, data suggest that follicle growth following IVD removal is not affected by pretreatment with MAP (Borges et al. 2008).

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In conclusion higher doses of MAP alone or lower doses of MAP in conjunction with EB are able to suppress growth of the dominant follicle in cattle. Although the injection of
EB at start of MAP treatment was potent in suppressing follicle growth, it did not induce luteolysis at midcycle.

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