The role of angiotensin II in the early stages of bovine ovulation

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Abstract

There is evidence that the renin–angiotensin system plays an important role in ovulation in cattle. Using an *in vivo* model, we investigated the role of angiotensin (Ang) II in bovine ovulation by injecting Ang II receptor antagonists into ovulatory follicles. Animals ($n=102$) were pre-synchronized and, when the follicles reached 12 mm, they were given the respective treatment and the cows received GnRH agonist (i.m.) to induce ovulation. The ovulation rate was significantly lower when 100 μ M saralasin (Ang II receptor antagonist) was intrafollicularly injected (14.3%) in comparison with saline solution (83.3%). Based on these results, a second experiment was carried out to determine the timing of Ang II's critical role in ovulation. Saralasin inhibited ovulation only when applied at 0 and 6 h (16.7 and 42.9% ovulation rate in the 0- and 6-h groups respectively), but not at 12 h (100%) following GnRH agonist treatment. To investigate the subtypes of Ang II receptors implicated in the LH-induced ovulation, losartan (LO; AT₁-Ang II receptor antagonist), PD123 319 (AT₂-Ang II receptor antagonist), LO+PD123 319, or saline were intrafollicularly injected when the cows were challenged with GnRH agonist. Ovulation was inhibited by PD123 319 and LO+PD123 319 (50.0 and 33.3% on ovulation rate respectively), but not by LO or saline solution (100% ovulation in both groups). From these results, we suggest that Ang II plays a pivotal role in the early mechanism of bovine ovulation via the AT₂ receptor subtype.

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Introduction

Ovulation is dependent on adequate luteinizing hormone (LH) release, which activates several established components of the ovulatory cascade (Espey 1980, Robker *et al.* 2000). The understanding of ovulatory pathways can be considered a new approach for infertility treatment and control ovulation in mammals (i.e. non-steroid contraceptives for women and bitches, and estrous cycle control in cows). However, the intrafollicular factors that initiate and control the ovulatory process are not well understood. Cyclooxygenase-2 has been identified as the key player that initiates the cascade of proteolytic activity required for tissue remodeling process during ovulation (Sirois 1994, Sirois & Dore 1997, Robker *et al.* 2000, Peters *et al.* 2004). In some tissues, Ang II induces an increase in expression of cyclooxygenase-2 and prostaglandins (Gimbrone & Wayne Alexander 1975, Hernández *et al.* 2002, Scheuren *et al.* 2002, Kim *et al.* 2005). The action of Ang II in the ovulatory process appears to differ between some species. Using an *in vitro* model, Yoshimura *et al.* (1993) showed that Ang II plays an essential role in inducing prostaglandin production

during ovulation in the rabbit. However, Husain *et al.* (1987) did not find the expected autoradiographic distribution of Ang II receptors in every developing follicle, suggesting that not all preovulatory follicles necessarily respond to exogenous or endogenous Ang II in the rat. Also in the rat ovary, Ang II receptors were not detected in preovulatory follicles with LH receptors (Daud *et al.* 1989). Using the microdialysis technique, Acosta *et al.* (2000) demonstrated that Ang II has a role in angiogenesis during ovulation, formation of the corpus luteum, and luteolysis in cattle. In addition to its vascular action, it was demonstrated that Ang II induces prostaglandin production in the rabbit ovary (Yoshimura *et al.* 1992, Kuji *et al.* 1996). Additionally, Ang II activity has been related to oocyte maturation, ovulation, and steroidogenesis in the rabbit (Yoshimura *et al.* 1992, 1993, Feral *et al.* 1995, Tanaka *et al.* 1995, Hayashi *et al.* 2000) and associated with resumption of meiosis (Giometti *et al.* 2005, Stefanello *et al.* 2006), steroidogenesis (Acosta *et al.* 1999), and follicular growth (Nielsen *et al.* 1994, Portela *et al.* 2006) in cattle.

At least two Ang II receptor subtypes, AT₁ and AT₂, have been identified and characterized (Bottari *et al.* 1993, DeGasparo *et al.* 1995, Gallinat *et al.* 2000). The

AT₁ receptor mediates a number of well-known Ang II effects on smooth muscle contraction, aldosterone secretion, and blood pressure regulation, while the AT₂ receptor has been shown to mediate the opposite effects and induce apoptosis (Yamada *et al.* 1996) and reproductive functions, including steroidogenesis, oocyte maturation, and ovulation (Kuji *et al.* 1996, Yoshimura *et al.* 1996). Yoshimura *et al.* (1996) inhibited ovulation using a specific AT₂ inhibitor (PD123 319) in rabbit ovaries perfused *in vitro*, but not using CV-11 974, a specific inhibitor of AT₁ receptors. PD123 319 is an AT₂ receptor antagonist with low affinity for AT₁ receptors, and specifically inhibits binding of Ang II at a concentration of 100 µM (Yoshimura *et al.* 1996). Losartan (LO) is an antagonist of AT₁ receptors with low affinity for AT₂ receptors (Chiu *et al.* 1989, Whitebread *et al.* 1989) and is of great therapeutic value for the control of blood pressure. In cattle, Ang II type 2 receptor was identified mainly in the dominant follicle by autoradiograms (Schauser *et al.* 2001), and its expression was positively correlated with follicular diameter (Nielsen *et al.* 1994). Recently, our group demonstrated that expression of AT₂ mRNA in granulosa cells is correlated with follicle health and estradiol content and is regulated by follicle-stimulating hormone (FSH), insulin-like growth factor-I, bone morphogenetic protein-7, fibroblast growth factor (FGF-7), and FGF-10 (Portela *et al.* 2006, Portela *et al.* 2007). We also showed that Ang II regulates protease nexin-1 (PN-1; inhibitor of proteases involved in extracellular matrix remodeling and follicle rupture) in granulosa cells via AT₂ receptor in cattle (Portela *et al.* 2006).

Since the requirement for Ang II in the ovulatory process is species specific and the *in vitro* receptor expression studies do not provide conclusive evidence that Ang II is essential for ovulation to occur, we designed experiments to investigate the hypothesis that Ang II is required to initiate the ovulatory process in cattle. The requirement for Ang II for the ovulatory process and the receptor subtype were investigated using an *in vivo* model. Specific inhibitors of Ang II receptors were injected into ovulatory follicles by an ultrasound-guided intrafollicular injection system to manipulate the follicular environment in cattle.

Results

Effect of Ang II receptor antagonist on ovulation

The first experiment was conducted to assess the role of Ang II in ovulation. Follicles that received saline ovulated at significantly higher rates (88.9%; 8 out of 9) when compared with those treated with saralasin (Ang II receptor antagonist; 38.5%; 5 out of 13; $P < 0.0342$; Fig. 1) after challenging the cows with gonadotropin-releasing hormone (GnRH) agonist. Nine cows (9 out of 22) were detected in estrus when treatments were applied. In the absence of estrus, the ovulation rates

were 14% (1 out of 7) and 83% (5 out of 6) in the saralasin and control groups respectively ($\chi^2 = 4.89$, 1 d.f., $P < 0.05$; Fig. 1). However, there was no difference in the ovulation rates when saline (100%; 3 out of 3) and saralasin (67%; 4 out of 6) were injected during estrus ($\chi^2 = 0.08$, 1 d.f., $P > 0.05$; Fig. 1). There was no difference between the mean diameters of the follicles injected with saralasin (13.0 ± 0.39 mm) and saline (13.7 ± 0.82 mm, $P > 0.05$). In addition, the mean diameter of the injected follicle was not different in cows that ovulated (13.7 ± 0.63 mm) when compared with those that did not ovulate (12.7 ± 0.32 mm; $P > 0.05$).

Effect of Ang II receptor antagonist during the process of ovulation

In order to evaluate whether Ang II is only a prerequisite for initiating the cascade of events or for the whole process that ultimately leads to ovulation, saralasin was intrafollicularly injected at 0, 6, and 12 h after GnRH challenge. The ovulation blocking effect of saralasin occurred when applied at 0 h (16.7%, 1 out of 6; $\chi^2 = 19.6$, 1 d.f., $P < 0.0001$) and 6 h (42.9%, 3 out of 7; $\chi^2 = 61.8$, 1 d.f., $P < 0.0001$), but not at 12 h (100%, 7 out of 7). There was no significant difference between the first two groups ($\chi^2 = 0.98$, 1 d.f., $P > 0.05$; Fig. 2). The follicular diameter (mean $\pm$ S.E.M.) was not different at the time of intrafollicular injection at 0 h (12.8 ± 0.41 mm), 6 h (13.9 ± 0.60 mm), and 12 h (14.3 ± 0.57 mm), or between cows that ovulated (14.2 ± 0.48 mm) when compared with those that did not ovulate (13.1 ± 0.37 mm; $P > 0.05$).

Figure 1 Ovulation rate following ultrasound-mediated intrafollicular injection of an angiotensin II receptor antagonist (saralasin) in cows. When the follicles reached 12 mm, they were given the respective treatment and the cows received GnRH agonist (100 µg gonadorelin acetate, i.m.) to induce ovulation. The cows were randomly chosen to receive an intrafollicular injection of saralasin (saralasin group, $n = 13$) or NaCl 0.9% solution (saline group, $n = 9$) before ($n = 13$) or after ($n = 9$) the onset of estrus. *Statistical difference between groups ($P < 0.05$).

Effect of AT₁ and AT₂ receptor antagonists on ovulation rate

The effect of Ang II receptor antagonists was assessed by intrafollicular injection of the Ang II type 1 receptor antagonist LO (DUP, 753) and/or type 2 receptor antagonist PD123 319. Ovulation rate was significantly reduced by PD123 319 ($P < 0.0001$), but not by LO (Fig. 3). A similar reduction in ovulation rate was observed when both LO and PD123 319 were applied to the same follicle. The diameters of the largest follicle (mean $\pm$ S.E.M.) when the antagonists were intrafollicularly injected were 13.1 ± 0.20 , 13.0 ± 0.30 , 12.8 ± 0.29 , and 13.0 ± 0.13 mm in the treatment (LO, PD, and LO+PD) and control groups respectively ($P > 0.05$). The follicular diameters were not different in cows that ovulated (13.1 ± 0.12 mm) when compared with those that did not ovulate (12.7 ± 0.24 mm; $P > 0.05$).

Discussion

The present studies tested the hypothesis that Ang II is required for initiating the ovulatory process. Our significant findings are: (1) the ovulation rate decreased when saralasin (Ang II receptor antagonist) was administered before estrus onset and (2) within a few hours after challenge with GnRH agonist; (3) the ovulation rate was reduced following the intrafollicular injection of the AT₂ receptor antagonist (PD123 319), but not after AT₁ receptor antagonist (LO) treatment. The present results provide strong evidence that Ang II is involved in initiating the ovulatory cascade by activating the AT₂ receptor. These findings complement an earlier study, which demonstrated an increase in Ang II concentration in follicular fluid

Figure 2 Ovulation rate following ultrasound-mediated intrafollicular injection of an angiotensin II receptor antagonist (saralasin) at 0, 6, or 12 h after GnRH agonist challenge (100 μ g gonadorelin acetate, i.m.). The cows ($n = 20$) had follicular diameter of at least 12 mm at intrafollicular injection. Different letters above bars indicate statistical difference between groups ($P < 0.05$).

Figure 3 Effect of angiotensin II type 1 (AT₁) and type 2 (AT₂) receptor antagonists on ovulation rate in cow. When the follicular diameter reached at least 12 mm, they were given the respective treatment and the cows received a GnRH agonist (100 μ g gonadorelin acetate, i.m.) to induce ovulation. The animals received an ultrasound-mediated intrafollicular injection of 0.9% NaCl (saline, $n = 6$), losartan (LO, 10 μ M; AT₁ receptor antagonist; $n = 6$), PD123 319 (PD, 10 μ M; AT₂ receptor antagonist; $n = 6$), or 10 μ M LO + 10 μ M PD ($n = 6$). Different letters above bars indicate statistical difference between groups ($P < 0.05$).

concomitantly with the onset of ovulation after the ovulatory LH surge (Acosta *et al.* 2000).

In this study, saralasin was used as a potent Ang II antagonist that blocks all known receptor subtypes (Kuji *et al.* 1996, Yoshimura *et al.* 1996). The saralasin concentration of 10 μ M used in this experiment inhibits binding of Ang II to its receptors (Kuji *et al.* 1996). Saralasin does not appear to be toxic in follicular cells, since cows that received a sequential FSH treatment and intrafollicular injection of saralasin in 7 mm follicles displayed follicular growth and ovulated (unpublished data). The high ovulation rate (67%), observed when saralasin was injected intrafollicularly after the onset of estrus, also rules out a possible toxic effect of saralasin on follicular cells. In addition, this peptide has been widely used to block the effect of the renin–Ang system or to study the action of Ang II, showing a high specificity for its receptors (Kuji *et al.* 1996, Yoshimura *et al.* 1996, Camargo & Saad 1999).

The exclusion of animals that did not meet the criteria of a minimum follicular diameter (12 mm) was based on the high ovulation rate of follicles larger than 12 mm obtained after LH exposure (Sartori *et al.* 2001). The high ovulation rate obtained in the control groups (14 out of 15 animals, 93%) demonstrates that the possible damage caused by the intrafollicular injection technique was not significant for final follicular development or ovulation, similar to other studies (Kot *et al.* 1995, Ginther *et al.* 2004). The success rate of the intrafollicular injection technique was 81.5% (66 out of 81 animals), similar to those previously reported (Kot *et al.* 1995, Ginther *et al.* 2004). Our results provide

further evidence that the ultrasound-guided intrafollicular injection system is a effective method to investigate the role of Ang II in the ovulatory process in cattle and supports previous studies demonstrating a physiological function of this peptide by i.p. injection in rabbits (Pellicer *et al.* 1988).

Interestingly, ovulation was inhibited when saralasin was intrafollicularly injected at the same time as GnRH treatment, but, in contrast, all follicles that received saralasin 12 h after GnRH treatment reached ovulation. These results indicate that Ang II has a role in the early stages of the ovulatory cascade. Further, Ang II induced ovulation in *in vitro* perfused rabbit ovaries, and its levels increased in follicular fluid after exposure to gonadotropins, suggesting an intrafollicular increase in renin-like activity in initiating the ovulatory process (Yoshimura *et al.* 1994). In cows, the levels of Ang II increase after LH surge and remain elevated throughout the ovulatory process (Acosta *et al.* 2000), and as such Ang II seems to be a key determinant in the ovulatory process. It could be speculated that Ang II triggers cyclooxygenase-2 to initiate the ovulatory process, which is a feasible ovulatory event that might lead to ovulation (Sirois 1994, Sirois & Dore 1997, Peters *et al.* 2004). Yoshimura *et al.* (1993) demonstrated that Ang II may induce ovulation in the rabbit ovary, at least in part, by stimulation of prostaglandin production.

The results from the current study suggest that Ang II acts through AT₂ receptor to stimulate the ovulatory process. A specific AT₂ receptor antagonist PD123 319 inhibited ovulation independently of the presence of the AT₁ receptor antagonist LO in this study. Saralasin inhibited ovulation nearly 100% and, in the third experiment, ~30% of follicles ovulated when the AT₂ receptor inhibitor (PD123 319) was used. These findings are in accordance with a competition assay described by Yoshimura *et al.* (1996), where saralasin inhibited specific binding of Ang II in a concentration-dependent manner, reaching 100% inhibition at 10⁻⁷ M, while specific inhibitors of Ang II receptors do not completely inhibit Ang II binding even at 10⁻⁴ M. We showed for the first time that intrafollicular injection of AT₂, but not AT₁, receptor antagonist *in vivo* inhibited ovulation in cattle. We are aware that the characterization of Ang II receptor subtype in bovine ovarian follicles has been described (Schauser *et al.* 2001); however, our findings provide additional support to *in vitro* studies, showing AT₂ receptor function *in vivo*. In rabbits, Yoshimura *et al.* (1996) induced ovulation and oocyte maturation in ovaries with Ang II via the AT₂ receptor subtype and inhibited ovulation with PD123 319 administration but not with CV-11 974, a specific inhibitor of AT₁ receptors. In the present study, PD123 319 was used because of its high affinity for AT₂ receptors, and the concentration used in this experiment (100 µM) caused complete inhibition of specific Ang II binding to AT₂ receptor in rabbit ovaries (Kuji *et al.* 1996). Taken together, these

data suggest that Ang II plays an important role in the ovulatory cascade via AT₂ receptors in cattle.

We recognize that we cannot exclusively attribute the effects of Ang II on the ovulatory cascade using the ultrasound-guided intrafollicular injection approach. However, we have previously demonstrated that AT₂ receptor mRNA levels are significantly higher in granulosa cells of estrogenic follicles when compared with non-estrogenic follicles, and AT₂ receptor mRNA is correlated with estradiol concentrations in follicular fluid (Portela *et al.* 2006). In addition, Ang II inhibits PN-1 expression, an inhibitor of proteases involved in extracellular matrix remodeling and follicle rupture, providing evidence that Ang II can regulate ovulation (Portela *et al.* 2006). Moreover, oocyte maturation, which is an event that occurs just before ovulation and after the LH surge, is mediated by Ang II (Giometti *et al.* 2005, Stefanello *et al.* 2006). Studies with laboratory animal species also support these results. Ovulation is inhibited by the administration of saralasin to *in vitro* perfused rabbit ovaries (Yoshimura *et al.* 1993). However, in rat, Ang II receptors were not detected in every developing follicle (Husain *et al.* 1987) and in preovulatory follicles with LH receptors (Daud *et al.* 1989), suggesting a species-specific effect of Ang II on ovulation.

Cattle provide an excellent model for studying the ovulatory process, since the follicular environment can be easily modified by ultrasound-guided intrafollicular injection (Kot *et al.* 1995, Ginther *et al.* 2004) and accurately monitored on a day-to-day basis by ultrasonography *in vivo* (Savio *et al.* 1988, Ginther *et al.* 1989, Evans & Fortune 1997). Using this *in vivo* model, we demonstrated that Ang II is essential for ovulation in cattle, since inhibition of Ang II by intrafollicular injection of saralasin led to inhibition of ovulation. These results also provide strong evidence that Ang II has a role in the early stages of the ovulatory cascade, acting as a key factor in the ovulatory process through the AT₂ receptor.

Materials and Methods

Inhibitors, hormones and supplies

The Ang II receptor antagonists, saralasin and PD123 319, were purchased from Sigma and LO (DuP 753) from Du Pont Merck Pharmaceutical Company. The ultrasound system, including scanner (Pie Medical Scanner 200) probes, biopsy, and needle guide, was provided by Pie Medical Equipment BV (Maastricht, The Netherlands). The controlled internal drug release (CIDR) devices were acquired from InterAg (Hamilton, New Zealand), estradiol benzoate from Genix (Anápolis, Brazil), sodium cloprostenol from Ouro Fino (Ribeirão Preto, Brazil), and equine chorionic gonadotropin (eCG) was from Syntex (Buenos Aires, Argentina). The GnRH agonist gonadorelin acetate was purchased from Tortuga (São Paulo, Brazil). The intrafollicular injection system needles were acquired from Becton,

Dickinson and Company (Franklin Lakes, NJ, USA). The Hamilton syringe was purchased from Sigma.

Animals and sequential ultrasound scanning

All experimental procedures using cattle were reviewed and approved by the Federal University of Santa Maria Animal Care and Use Committee (ACUC). One hundred and two cycling, non-lactating, mature Hereford cows with a body condition score of 3 or 4 (scale from 1-thin to 5-obese) were pre-synchronized with CIDR (the insertion of the vaginal device was defined as day 0) for 9 days, estradiol benzoate (5 mg, i.m., on day 0), sodium cloprostenol (125 µg, s.c., on day 6), and eCG (400 IU, i.m., on day 6). After CIDR device removal, the cows were checked twice a day for estrus behavior. Follicular growth was monitored daily through transvaginal ultrasonography by a single operator using an ultrasound equipped with a sectorial 7.5 MHz transducer. Only cows that reached a minimum follicular diameter of 12 mm after CIDR device removal were included in this study ($n=81$).

Ultrasound-guided intrafollicular injection procedures

The intrafollicular injections were guided by ultrasound equipped with a 7.5 MHz vaginal probe. A system with two sterile needles was adapted from Kot *et al.* (1995). The double-channeled needle system consisted of an inner sterile 25-gage needle attached to 35 cm long cannula and an outer 20-gage needle coupled to a needle guide. To the opposite end of the cannula, a Hamilton syringe was attached. Epidural anesthesia was induced, and perineal cleaning was performed before injections. The transducer was coupled to a biopsy guide (Pie Medical Equipment BV), coated with lubricant, and placed in the vaginal fornix. The course of the echogenic needle was observed on the ultrasound display, which allowed us to reach the follicle of interest. The ovary was manipulated so that the needle entered the follicle via penetration of the ovarian stroma at the base of the follicle. The needle path to the injected follicle contained ovarian stroma and no additional follicles or corpus luteum.

Immediately before injection, the inner needle system was washed consecutively with 70% alcohol and sterile 0.9% NaCl solution and filled with appropriate treatments, and the system was set-up. When the ovary and follicle of interest were in position, the outer needle was advanced until the image of its tip became visible on the screen, ~1 cm from the follicle. At this moment, a second operator pushed the inner needle forward until the image of the needle tip was visible within the follicle. Treatments were then injected into the follicle. Swirling of the fluid entering the follicle indicated that the injection was successful. The amount of Ang II receptor antagonists for the injection was calculated based on the volume of follicular fluid to obtain an adequate final concentration inside the follicle. The follicular fluid volume was estimated by the linear regression equation $V = -685.1 + 120.7D$ ($P=0.0001$), determined in a pre-experiment, where V corresponds to the estimated follicular volume and D to the diameter of the follicle to be injected. The probe and needles were withdrawn immediately after injection

to minimize pressure on the newly punctured follicle. Follicular diameter was measured again 2 h after intrafollicular injection. Cows were excluded from the experiment if the reduction in follicular diameter was > 2 mm.

Experiment 1: effect of angiotensin II (Ang II) receptor antagonist on ovulation

Twenty-five cows were randomly assigned to two groups to receive an intrafollicular injection of 100 µM saralasin (a potent Ang II inhibitor that blocks all known Ang II-receptor subtypes; saralasin treatment group; $n=13$) or 0.9% NaCl (saline control group; $n=12$). The follicles were intrafollicularly injected when they reached the size of 12 mm. The amount of saline and saralasin for the injection was calculated as described above, to obtain a final concentration of 10 µM saralasin inside the follicle. The experiment was performed in two replicates (10–15 cows per replicate). Three cows were discarded from the control group because the follicular diameter was reduced by more than 2 mm after intrafollicular injection. The cows were challenged with GnRH agonist (100 µg gonadorelin acetate, i.m.) just after intrafollicular injections. The injected follicle was monitored by ultrasound examination every 24 h until ovulation or atresia. Ovulation was presumed when the treated follicle was not present between two assessments, and the corpus luteum was subsequently formed. The absence of ovulation for a period of 48 h, associated with a gradual decrease in follicular diameter, was characterized as follicular atresia.

Experiment 2: effect of Ang II receptor antagonist during the process of ovulation

Twenty-five cows were randomly assigned to three groups to receive an intrafollicular injection of 100 µM saralasin (saralasin treatment group) under similar conditions as in the previous experiment. The volume of saralasin to be injected was estimated as described above. The cows received saralasin at 0 (0 h group; $n=6$), 6 (6 h group; $n=7$), or 12 h (12 h group; $n=7$) following GnRH agonist injection. The 'time 0' was considered when the follicles reached 12 mm. The experiment was performed in three replicates (seven to ten cows per replicate). The injected follicle was monitored by ultrasound examination every 24 h until ovulation or atresia, as described in the first experiment. Five cows were discarded from the experiment because the follicular diameter was reduced by more than 2 mm after intrafollicular injection.

Experiment 3: effect of AT₁ and AT₂ receptor antagonists on ovulation rate

Thirty-one cows divided into four groups were treated with GnRH agonist (100 µg gonadorelin acetate, i.m.) and randomized in a 2×2 factorial design: intrafollicular injection of 100 µM LO group; specific inhibitor of AT₁ receptors), 100 µM PD123 319 (PD group; inhibitor of AT₂ receptors), 100 µM PD123 319+100 µM LO (PD+LO group), or 0.9% NaCl (saline group). The moment of intrafollicular injection was determined when the follicle reached 12 mm, and the four

treatments were tested in three replicates. The injected volume was based on the follicular fluid volume (estimated as described above) to obtain a final concentration of 10 µM of each inhibitor. Seven cows were discarded from the experiment because the follicular diameter was reduced by more than 2 mm after intrafollicular injection.

Statistical analysis

Validation of the results for the three experiments was performed by analysis of variance of follicular diameter at the time of treatment using the class variables treatment, ovulation, and treatment versus ovulation interaction as factors. The ovulation rate was compared between the different treatments in a statistical model for categorical variables using PROC CATMOD (Categorical Data Analysis Procedures), and differences between groups were analyzed by contrasts. All analyses were performed with the SAS statistical program (SAS Institute, Cary, NC, USA) with the level of significance set at 5%.

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