Synchronization of time of ovarian follicular development in llamas (*Lama glama*) using a protocol based on GnRH and PGF$_{2\alpha}$

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A B S T R A C T

The aim of this study was to evaluate the effectiveness of a protocol based on GnRH and PGF$_{2\alpha}$ to synchronize the emergence of a new wave of ovarian follicular development in llamas and, therefore, when a new dominant follicle develops. Llamas ($n = 18$) were assigned to growing, mature or regressing follicle groups according to the phase of the follicular wave at the beginning of treatment. The protocol was initiated with a GnRH analogue (GnRHa) injection on Day 0 followed 7 days later with a d-cloprostenol injection and a second GnRHa injection on Day 10. Ovulation rate after the first GnRHa treatment, day of new follicle emergence, mean plasma progesterone concentration and percentage of animals with a newly developed dominant follicle ≥ 7 mm on Day 10 were evaluated. Ovulation rate after the first GnRHa was less in the regressing than mature and growing follicle groups and new follicular wave emergence occurred earlier in the regressing follicle group than in the other two groups. Mean plasma progesterone concentration in females that had ovulations after the first GnRHa injection was similar. The percentage of animals that had a new follicle ≥ 7 mm on Day 10 was not different among groups and the overall percentage was 66.6%. The total synchronization rate for development of a new wave of follicular development on Day 10 was greater in females having ovulations after the first GnRHa injection than in those that did not have ovulations. In conclusion, the protocol used in the present study was useful for synchronizing ovarian follicular development in 66% of the llamas regardless of the phase of the follicular wave development at the beginning of treatment.

1. Introduction

Llamas are an induced ovulating species requiring copulation to induce the ovulatory process (San-Martin et al., 1968; Bravo et al., 1991). Unmated females have waves of follicular growth and regression that overlap with the preceding wave of follicular development (Cavilla et al., 2013) and are sexually receptive most of the time (England et al., 1971). The capacity for ovulation from follicles depends on the size of the follicle at the time of mating: there is generally ovulation if follicles are larger than 7 mm in diameter whereas there is not from smaller growing follicles or regressing follicles. These latest follicles become luteinized without ovulation occurring (Bravo et al., 1991). Consequently, the close association between estrous behavior and ovulation that exists in most domestic animals does not occur in llamas. In addition, to improve pregnancy rates after mating it is important to mate or inseminate the female when there is growing or early static growth of the dominant follicle which contain oocytes with a greater capacity for fertilization (Vaughan et al., 2003). Many matings, therefore, do not result in pregnancies due to failure to induce

ovulation or the presence of an aged oocyte in the follicle from which ovulation occurs. The development of a protocol to synchronize the time of emergence of a new wave of follicular development in llamas and, therefore, allow the prediction of the time when a new dominant follicle is present, would enable for scheduling fixed timed matings or inseminations as well as to synchronize groups of females for embryo transfer programs.

In cattle, a estrous synchronization protocol, known as ovsynch, based on two injections of gonadotropin releasing hormone analogue (GnRHa), 8 or 9 days apart, in association with a prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) injection has been used successfully for timed breeding. An injection of GnRHa is administered at a random stage of the estrous cycle, which causes ovulation from or luteinization of the cells of large follicles present in the ovary and synchronizes the emergence of a new wave of ovarian follicular development. An injection of $PGF_{2\alpha}$ is administered 7 days later to induce the regression of the corpus luteum. Time of ovulation from the newly developed dominant follicle is synchronized by the second injection of GnRHa given 2 days later (Pursley et al., 1995). In camels, a similar protocol (GnRHa injection followed 7 days later by an injection of a $PGF_{2\alpha}$ analogue and a second GnRHa injection 7 days later) was evaluated with results indicating that this protocol was the most effective to synchronize time of ovulation at a fixed time interval of 14 days after treatment in this species (Skidmore et al., 2009).

Previous studies in llamas indicate that administration of GnRHa induces ovulation when a follicle larger than 7 mm is present (Ratto et al., 2006). In addition, in another study the llama corpus luteum was sensitive to a synthetic $PGF_{2\alpha}$ analogue after day 5 subsequent to induction of ovulation (Bianchi et al., 2012).

Thus, the aim of the present study was to evaluate the effectiveness of a protocol based on GnRH and $PGF_{2\alpha}$ injections to synchronize the time of emergence of a new wave of ovarian follicular development in llamas and, therefore, to allow the prediction of time when a newly developed dominant follicle is present in a group of llamas regardless of the phase of the follicular wave at the beginning of treatment. It was hypothesized that the first GnRHa injection would induce ovulation from any follicle $\geq$ 7 mm, thus, initiating a new wave of ovarian follicular development or administration would occur at a time when a new wave of ovarian follicular development was emerging spontaneously. If a corpus luteum results from the initial injection of GnRHa, the d-cloprostenol will induce luteolysis 7 days later, and a new dominant follicle $\geq$ 7 mm will be present 3 days later.

2. Materials and methods

2.1. Experimental design

Field studies were performed in compliance with animal welfare regulations of the Faculty of Veterinary Sciences, Universidad Nacional del Centro de la Provincia de Buenos Aires where activities were conducted. Facilities where the study was conducted are located in Tandil, Argentina, at 37°S, 60°W. Animals were confined in pens isolated from males and fed pasture hay and water *ad libitum*.

Llamas ($n = 18$) were divided into three groups according to the phase of the follicular wave development: Growing follicle group ($n = 6$): follicles between 4 and 7 mm of diameter that increased in size between two consecutive measurements; mature follicle group ($n = 6$): follicles between 8 and 15 mm and regressing follicle group ($n = 6$): follicles with decreasing diameter between at least two consecutive measurements and without a growing follicle $\geq$ 4.5 mm in any of the ovaries. The phase of the follicular wave development was determined by trans-rectal ultrasonography during 3 consecutive days before the beginning of the study as previously reported by Cavilla et al., 2016.

The day of treatment initiation was considered Day 0 and the protocol was initiated with an injection of a GnRHa (8.2 µg i.v., Buserelin acetate, Gonaxal®, Biogénesis Bagó, Argentina) followed 7 days later with an injection of d-cloprostenol (synthetic analogue of $PGF_{2\alpha}$, 105 µg i.m., Enzaprost DC, Biogénesis Bagó, Argentina) and a second GnRHa injection 3 days later (Day 10) only in those animals that had a follicle $\geq$ 7 mm, at a growing or early mature phase.

All llamas were examined daily from 3 days before the beginning of treatment to Day 10 by tran-srectal ultrasonography (Mindray, DP 6600 Vet, with 5.0/7.5 variable traducer probe) to assess diameters of the follicles and the corpus luteum which was estimated averaging two measurements of the diameter at right angles to each other (Moreira et al., 2000).

Occurrence of ovulation was assessed based on ultrasonographic visualization of the corpus luteum and further confirmed by the progesterone profiles. Day of emergence of the newly developing dominant follicle, retrospectively determined, was considered the day it first measured $\geq$ 3 mm in diameter (Cavilla et al., 2013), and the new dominant follicle was defined as the one that emerged after beginning of the treatment with a diameter $\geq$ 7 mm at the growing or early mature phase by Day 10.

Blood samples were collected by jugular venipuncture on Days 0, 2, 4, 6, 7 and 8 and daily after the day of the second injection of GnRHa for 12 days. Samples were centrifuged and plasma was stored at -20 °C until hormone assays were performed.

2.2. Hormone determinations

Progesterone concentration was quantified using a RIA kit (IM 1188, Beckman Coulter, Immunotech, Czech Republic). Serially diluted llama plasma samples containing relatively greater progesterone concentrations produced curves parallel to the standard curve. The sensitivity of the assay was 0.10 ng/ml and the intra-assay coefficient of variation was less than 6% for concentrations between 0.10 and 53 ng/ml.

Table 1
Mean follicular diameter at beginning of treatment, percentage of animals that had ovulations after the first GnRHa injection, mean day of the emergence of the new wave of follicular development and percentage of animals with a new, follicle ≥ 7 mm at growing or early mature phase on day 10 of treatment. Values with different superscripts within a column are different.

Group	Mean follicular diameter (mm)	Ovulation rate to first GnRHa (%)	Emergence new wave (days)	Ovulatory follicle on day 10 (%)
Growing follicle	6.15 ± 3.30[a]	83.3 (5/6)[ac]	2.3 ± 0.25 (4/6)[a]	66.6 (4/6)[a]
Mature follicle	10.75 ± 0.97[b]	100 (6/6)[ab]	3.0 ± 0.0 (5/6)[a]	83.3 (5/6)[a]
Regressing follicle	10.38 ± 1.09[b]	33.3 (2/6)[c]	1.3 ± 0.3 (3/6)[b]	50 (3/6)[a]

2.3. Statistical analysis

Data were analyzed using an ANOVA test followed by the Tukey's Test to detect differences between means in the diameter of the largest follicle at the time of the first GnRHa injection, day of the emergence of the newly developed dominant follicle and follicular diameter on Day 10. Due to the small number of animals that had ovulations after the first GnRHa injection in the regressing follicle group, mean corpus luteum diameter the day of d-cloprostenol injection and mean plasma progesterone concentration from Day 0 to 24 hours after the day of d-cloprostenol injection was evaluated between growing and mature follicle groups. The proportions of llamas having ovulations after the first GnRHa injection, percentage of animals with a newly developed dominant follicle ≥ 7 mm on Day 10 of treatment in the different groups and total follicular development synchronization rate on Day 10 were compared using the Pearson´s chi-square test.

All statistical analyses were conducted using the Infostat Professional 2017 software package. Data are presented as mean ± S.E.M., and differences were considered to be significant when $P < 0.05$.

3. Results

Data regarding mean diameter of the largest follicle on Day 0, ovulation rate after the first GnRHa injection, mean day of the emergence of the newly developed dominant follicle, and percentage of animals with a follicle from which ovulation occurred by day 10 are included in Table 1. The mean diameter of the largest follicle on Day 0 was smaller in the growing follicle group than mature and regressing follicle groups ($P = 0.0029$).

Frequency of ovulation following the first GnRHa injection was less in the regressing than mature follicle group ($P = 0.01$) and tended to be less than in the growing follicle group ($P = 0.079$). The overall ovulation rate after the first GnRHa injection was 72.2% (13/18).

The mean day of emergence of the newly developed dominant follicle was similar between the growing and mature follicle groups. In the regressing follicle group, the emergence of the new wave of follicular development occurred earlier ($P = 0.0009$) than in the other groups (Table 1; Fig. 1).

Mean corpus luteum diameter the day of d-cloprostenol injection did not differ ($P = 0.35$) between the growing follicle (12.13 ± 0.31 mm) and mature follicle group (13 ± 0.68 mm). In the growing and mature follicle groups, mean plasma progesterone concentration of the females from which ovulations occurred after the first GnRHa injection were similar on all days
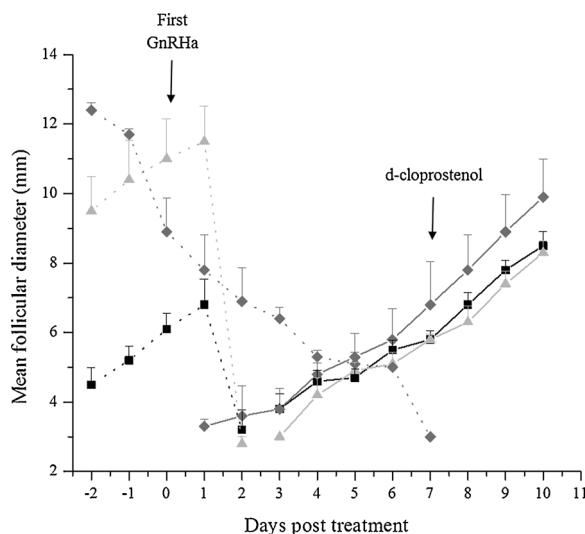

Fig. 1. Mean diameter of the largest follicle from 2 days before beginning of treatment until day 10 post treatment; ■ growing follicle group (n = 6); ▲: mature follicle group (n = 6) and: ♦regressing follicle group (n = 6); Dotted and solid line represent previous and new follicular wave.
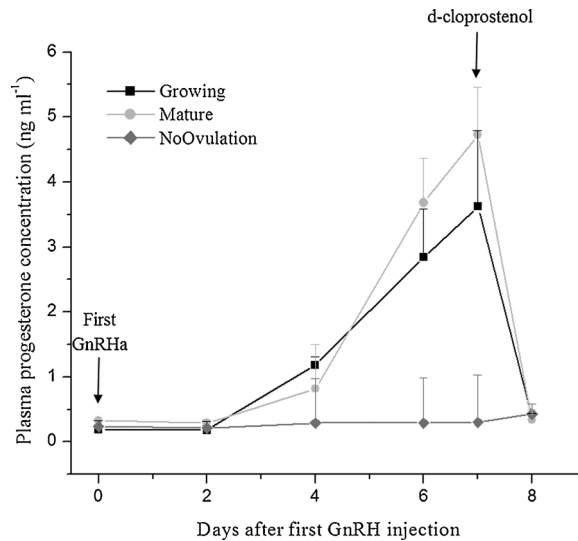
Fig. 2. Mean plasma progesterone concentration from the beginning of the treatment until Day 8 (24 hours after d-cloprostenol injection); Black line: growing follicle group ($n = 5$) and Light gray: mature follicle group ($n = 6$); Dotted line represents animals that did not have ovulations after the first GnRHa injection.

evaluations occurred ($P = 0.85$) and a similar pattern was observed in the two animals from which there were ovulations in the regressing follicle group. Mean plasma progesterone concentration in those animals that did not have ovulations after the GnRHa injection were less than 1 ng/ml during the entirety of the study (Fig. 2).

All the animals with a corpus luteum on Day 7 responded to the d-cloprostenol injection, having plasma progesterone concentrations of less than 1 ng/ml, 24 hours after the luteolytic injection (Fig. 2).

The percentage of animals that developed a new wave of follicular development and had a follicle ≥ 7 mm at the growing or early mature phase, on Day 10 was not different among groups ($P = 0.47$) and the overall percentage was 66.6% (12/18). In these animals, mean follicular diameter on Day 10 was 8.5 ± 0.41; 8.3 ± 0.34 and 9.9 ± 1.10 mm in the growing, mature and regressing follicle groups and these values were not different ($P = 0.17$). The total synchronization rate of ovarian follicular development on Day 10 was greater in females with ovulations after the first GnRHa injection than in those that did not have ovulations as a result of this treatment (92% compared with 20%) ($P = 0.0022$).

Mean plasma progesterone concentration after the second GnRHa injection is depicted in Fig. 3. Plasma concentration of progesterone increased to greater than 1 ng/ml on Day 5, peaked on Day 7 and started to decrease by Days 8–10 with the basal concentration occurring between Days 11 and 12 (Fig. 3).

In six animals, the treatment failed to induce development of a follicle at a growing or early mature phase (≥ 7 mm). In two of these animals of the regressing follicle group, the emergence of the new wave of follicular development started before the first GnRHa injection and the mature follicles of each female by Day 10 were at the late mature phase, thus, these follicles were not considered newly developed dominant follicles. In the other two animals (one of the growing follicle group and one of the mature follicle group),
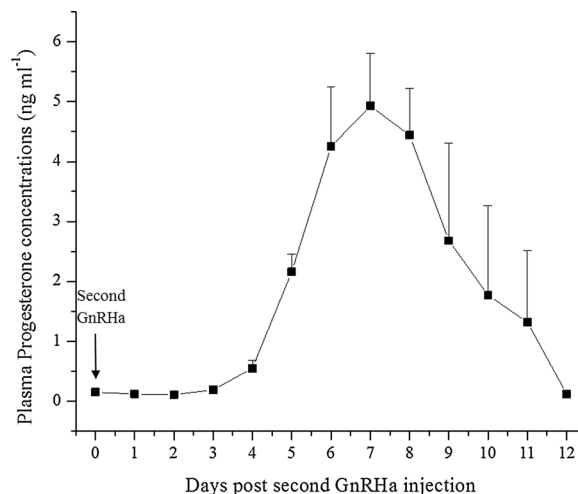

Fig. 3. Mean plasma progesterone concentrations after the second GnRHa injection in all animals with an ovulatory follicle on day 10 of treatment.

the emergence of the new wave of ovarian follicular development was observed on Day 3 but the follicular growth was slow and the diameter of the largest follicle was not ≥ 7 mm on Day 10 post treatment. In one animal of the growing follicle group and in one of the regressing follicle group, that did not have ovulations after the first GnRHa injection, the diameter of the largest follicle was 9 and 10 mm, respectively, on the day of d-cloprostenol injection. At 48 hours later, the dominant follicle had disappeared and 7 days later a corpus luteum was detected and plasma progesterone concentration was greater than 2 ng/ml, in both animals. Consequently, none of these animals received the second GnRHa injection.

4. Discussion

Although different protocols to control ovarian function have been developed in llamas (Aba et al., 2000; Chaves et al., 2002; Ratto et al., 2003; Cavilla et al., 2016), to our knowledge, this is the first report where the effectiveness of a protocol based on GnRH and $PGF_{2\alpha}$ injections was evaluated. Moreover, the development of a new dominant follicle, from which ovulation would occur on a specific day, in a group of llamas regardless of the stage of follicular wave development at the beginning of the treatment was evaluated.

The phase of follicular wave development at the initiation of this protocol resulted in differences in the ovulation rate that occurred as a result of the first GnRHa injection. The significant percentage of animals that had ovulations after the first GnRHa injection is a finding that is partially consistent with previous reports in camelids where there were indications that ovulations can occur from follicles in the growing phase with a diameter between 6 and 7 mm and in mature phase (≥ 7 mm) (Bravo et al., 1991). In the present study, it was also observed that ovulations could occur from follicles with a diameter of 5.5 mm with these follicles increasing in diameter to 6.5 mm 1 day later and ovulations occurring from these follicles by 48 h after the first GnRHa injection. Inconsistent with these findings, Bravo et al (1991) have reported that ovulations will not occur from follicles with a diameter of 6 mm. Results from this same previous study indicated there were not ovulations in females with regressing follicles but instead these follicles became luteinized in response to copulation. In the present study, however, a lesser number of animals with regressing follicles had ovulations (33.3%) or the follicles continued to regress without luteinization (66.6%). This observation is consistent with findings in a previous study in which there were not ovulations in response to GnRH in heifers with large follicles when these follicles were in regressing phase (Martinez et al., 1999).

The time of the emergence of the new wave of follicular development from which a new dominant follicle developed was dependent on the stage of the previous follicular wave on Day 0 with the interval between the initiation of the treatment to follicular wave emergence being shorter in the regressing follicle group. In the three females in which there was synchronization of the wave of follicular development that had regressing follicles on Day 0, the emergence of the new wave of follicular development occurred 1 or 2 days after the initiation of the protocol, due to the overlapping of the follicular waves. This observation is consistent with the findings in a previous study where follicular development in anovulatory llamas was characterized by the emergence of successive waves that overlapped to varying degrees (Cavilla et al., 2013). Values for the mean day of the emergence of the new wave of follicular development in llamas of the growing or mature follicle group are consistent with results found in camels in which the new wave of follicular development emerged at around 3 days after the GnRH injection (Nikjou et al., 2008).

The observation that all animals with a corpus luteum on Day 7 responded to the injection of a $PGF_{2\alpha}$ analogue with plasma progesterone concentrations decreasing to less than 1 ng/ml 1 day later is consistent with findings in a previous study that indicated the corpus luteum of llamas is completely sensitive to $PGF_{2\alpha}$ after day 5 from induction of ovulation (Bianchi et al., 2012).

The finding that two llamas with a mature follicle on Day 7 had ovulations after the d-cloprostenol injection was completely unexpected. It has been reported in previous studies with immature ewes and prepubertal heifers that $PGF_{2\alpha}$ is associated with LH release and ovulation as a consequence of a luteolysis–independent mechanism (Bono et al., 1980; Leonardi et al., 2012).

The protocol evaluated in the present study was efficient in synchronizing the development of a new dominant follicle at the growing or early mature phase (≥ 7 mm) of follicular development in 66% of the llamas on Day 10. From previous studies in camelids, it was reported that these follicles produce the greatest concentrations of estradiol (Cavilla et al., 2013) and pregnancy rates are greater when mating occurs in presence of highly estrogenic follicles (Vaughan et al., 2003). In the present study, although a similar percentage (72%) of animals had ovulations after the first GnRHa injection, the timing of initiation of a wave of follicle development was not synchronized. These animals likely had follicles from early growing to late regressing phases, therefore, there were different concentrations of estradiol and the oocytes yielded would likely have been of variable quality as previously reported (Vaughan et al., 2003).

In the present study, llamas that had ovulations after the first GnRHa injection had a greater rate of synchronization of ovarian follicular development than females that did not have ovulations (92% compared with 20%). Similarly, from results of a previous study, evaluating the ovsynch protocol in dairy cows it was suggested that a lesser rate of ovulation after the first GnRH injection resulted in a lesser synchronization rate of ovarian follicular development after the second GnRH injection (Vasconcenlos et al., 1999). A previous study in llamas has demonstrated that development of follicular waves generally overlap indicating that, a follicle from which ovulation can occur in response to the first GnRHa injection would usually be present in all animals (Cavilla et al., 2013), although the quality of the oocytes yielded would be highly dependent of the phase of the follicles at the time of treatment induction. Thus, the use of this protocol would allow for development of dominant follicles containing a healthy oocyte at a known time after treatment in at least 66% of the llamas.

5. Conclusions

In conclusion, use of the protocol of the present study resulted in synchronization of the timing of ovarian follicular development in llamas that was highly dependent on the number of females that had ovulations after the first GnRHa injection. There were a lesser percentage of ovulations in animals with regressing follicles if there was not a growing follicle at the beginning of the treatment. Emergence of the new wave of follicular development occurred between days 1–3 after initiation of treatment. The 66% of animals that had a synchronized stage of follicular development when using this protocol developed a new dominant follicle that was at a growing or early mature phase 10 days after the beginning of treatment. These follicles would be expected to contain a healthy oocyte with the capacity for fertilization to occur after mating. The efficacy of this protocol under field conditions and its effect on pregnancy rates remains to be elucidated.

Funding

Conflict of interest statement

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this article.

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