



# Plasma steroid profiles and ovarian response in llamas treated with eCG for superovulation combined with exogenous progesterone during early luteal phase

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## ABSTRACT

The objective of this study was to evaluate the effects of plasma progesterone (P<sub>4</sub>) concentrations during eCG-ovarian follicular superstimulatory treatment performed in early luteal phase and estradiol concentrations during peri-ovulatory period on ovarian response, number and embryo quality. On Day -2, females (n = 75) having a follicle ≥7 mm were treated with GnRH to induce ovulation. On Day 0, females that had ovulations (n = 54) were treated with 1000 IU eCG and were assigned to one of two treatments: (1) intravaginal device (ID) containing 0.5 g P<sub>4</sub> (P<sub>4</sub> group) and (2) no ID (Control group). On Day 5, females were administered PGF<sub>2α</sub> and the ID was removed. On Day 7 and 8, females were mated and embryo recovery was performed 7 or 8 days later. Blood samples were collected from Day 0 to 9. Number (± SD) of follicles ≥7 mm on day of mating was greater (P = 0.04) in the control (9.7 ± 4.2) than P<sub>4</sub>-treated (6.7 ± 4.9) group; number of corpora lutea did not differ (5.5 ± 3.1 and 5.2 ± 3.4 respectively). Ovulation rate was greater (P < 0.01) in the P<sub>4</sub>-group (77.4%; 130/168) than control group (53.3%; 135/253). Number of embryos with an excellent grade (grade 1) tended to be greater (P = 0.07) in the P<sub>4</sub>-group (82.4%; 42/51) than control group (65.4%; 36/55). It was concluded that supplementation with exogenous P<sub>4</sub> during eCG treatment in early luteal phase inhibits excessive follicular growth, increases ovulation rate and improves embryo quality.

## 1. Introduction

Multiple ovulation and embryo transfer (MOET) is an important technique for genetic improvement of many animals used for food and fiber production. Variability and poor ovarian responses of donors to superovulatory treatments are the primary limiting factors affecting the success with use of the MOET technology for genetic improvement programs in llamas. The use of ultrasonography has provided insights to some of the mechanisms regulating ovarian follicular development and regression, however, variation among donors is still one of the most limiting aspects in commercial embryo transfer enterprises (Ratto et al., 2013). Although the recent development of protocols that control follicular wave emergence have not eliminated the variability in superovulatory response in South American camelids (Ratto et al., 2013; Vaughan et al., 2013), the use of this technology allows the natural mating of a large number of donor llamas at a fixed-time.

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Several approaches have been developed to manipulate the timing of follicular wave development so that ovarian superstimulation treatments of follicular development are generally performed during: i) a period when there is treatment with an intravaginal progesterone/progestin device (Aller et al., 2002; Huanca et al., 2009) or ii) during a natural luteal phase by inducing ovulation with human chorionic gonadotropin (Bourke et al., 1995) or luteinizing-hormone (Huanca et al., 2009). In general, protocols for ovarian follicular superstimulation consist in the administration of follicle-stimulating hormone (FSH), equine chorionic gonadotropin (eCG) or combinations of the two (Correa et al., 1997) and are designed to initiate the treatment at the time of follicular wave emergence avoiding the suppressive effect of a dominant follicle (Carretero et al., 2010).

Results of several studies in sheep indicated a variability in ovarian response related to differences in superovulatory follicle treatments (Rubianes et al., 1999), FSH/LH ratio and dose numbers of p-FSH (D'Alessandro et al., 2005), follicular status at time of treatment initiation (Veiga-Lopez et al., 2005), presence of corpus luteum during treatment (Gonzalez-Bulnes et al., 2005) and reproductive seasonal changes (Gonzalez-Bulnes et al., 2003). In cattle, however, the administration of exogenous gonadotropins produce an important endocrine imbalance with concurrent abnormal egg and embryo development, premature ovulation or development of luteal cells and follicular cysts (Callesen et al., 1986). These problems in response to superovulatory treatments were more frequently encountered in eCG as compared to FSH-treated cows, due to eCG being a hormone with a complex glycoprotein structure that has both FSH and LH activity (Murphy et al., 1984) with a half-life of 40 h in cattle. The hormone continues to be present for 10 days in the blood of cattle (Mapletoft et al., 2002) with the relatively longer half-life than most hormones being due to terminal sialic acid residues of its N- and O-linked saccharide chains (Martinuk et al., 1991). The use of eCG has been greater than FSH for superovulatory treatment in embryo transfer programs with llamas due to its lesser commercial cost than FSH.

In llamas, Ratto et al. (2003) reported that the emergence of a new follicular wave occurred 2 days after LH treatment to induce ovulation. If the gonadotropin treatments are initiated around the time when there is initiation of development of a new follicular wave, the follicles grow when there is less systemic progesterone ( $P_4$ ) concentration, due to the presence of the peri-ovulatory corpus luteum (CL). In sheep, Cuadro et al. (2018) observed that  $P_4$  priming when there was follicular growth during the first follicular wave following ovulation improved the fertilization rate and the embryo yield in superovulated females and the main improvement was in embryo quality.

In cattle, the superovulatory response was less when gonadotropin treatment was initiated when peripheral  $P_4$  concentrations were relatively less as compared with those during the mid-luteal phase of the estrous cycle (Goto et al., 1988). In addition, in beef cows when there was superstimulated follicular development during the first follicular wave (relatively less  $P_4$ ), embryo quality was greater than when supplemental  $P_4$  was added to the treatment protocols (Nasser et al., 2011; Rivera et al., 2011). In a study in llamas (Huanca et al., 2009), however, there were no apparent differences in the number and embryo quality between females treated with eCG alone or with eCG plus a progestin-releasing intravaginal sponge.

To our knowledge there are no reports in llamas regarding the effect of eCG treatments on ovarian response and embryo yield in llamas when treatments for superovulation occur when there are peri-ovulatory plasma  $P_4$  and estradiol-17 $\beta$  ( $E_2$ ) concentrations. Preliminary results of this experiment were published in a previous congress proceedings (Aller et al., 2015b).

The present study was designed to test the hypothesis that the ovarian response and number and quality of embryos would be affected when eCG treatments for superovulation occurred when there are peri-ovulatory plasma  $P_4$  and estradiol-17 $\beta$  ( $E_2$ ) concentrations. The objectives of the present study, therefore, were to evaluate the effects: i) of administration of exogenous  $P_4$  by means of an intravaginal device during the eCG-superstimulatory treatment performed for follicular development in early luteal phase and ii) when there were peri-ovulatory plasma  $E_2$  concentrations on ovarian response and the number and embryo quality.

## 2. Materials and methods

### 2.1. Experimental conditions

This research was conducted during the breeding season (January to March) at the Abra Pampa Experimental Station of Altitude of the National Institute of Agricultural Technology (INTA) located in the province of Jujuy (22° 49' S, 65° 47' W; 3484 m above sea level) in the High Andean plateau of north-west Argentina. The animals were maintained in a native pasture of "chillagua" (*Festuca scirpifolia*) and water was provided *ad libitum*.

All animals were managed in accordance with procedures approved by the Committee for the Care and Use of Experimental Animals of the National Institute of Agricultural Technology of Argentina.

### 2.2. Animals and treatments

Females weighed  $104.3 \pm 5.8$  kg (mean  $\pm$  SD), with a body condition score of  $4.5 \pm 1.3$  (1 = emaciated to 9 = obese, Richards et al., 1986) determined by palpation over spinous processes, transverse processes, hip bones and ribs. A total of 86 non-lactating females were evaluated by clinical examination and trans-rectal ultrasonography (Honda HS 101 V, 5-MHz linear-array transducer, Japan) of the reproductive tract to detect any abnormalities or pregnancy. On Day -2, non-pregnant llamas having an ovarian follicle  $\geq 7$  mm in diameter ( $n = 75$ ) were treated with GnRH analogue (100  $\mu$ g of gonadorelin i.m., Gonasyn GDR®, Zoetis, Argentina) to induce ovulation. On Day 0, ultrasonic examinations of the ovaries were performed to confirm the occurrence of ovulation. Ovulation was defined as the sudden disappearance of a large follicle that was detected during the previous examination. Females that had ovulations in response to GnRH were randomly assigned in equal numbers ( $n = 27$ ) to one of two treatment groups: (1) intravaginal device (DIB 0.5°, Zoetis, Argentina) containing 0.5 g of progesterone ( $P_4$  group) and (2) no intravaginal device was

inserted (Control group). To stimulate multiple ovarian follicular development the females were treated with equine chorionic gonadotropin (eCG, Novormon 5000<sup>®</sup>, 1000 IU, i.m., Zoetis, Argentina). On Day 5, all females were treated with an i.m. injection of analogue of prostaglandin F<sub>2α</sub> (PGF<sub>2α</sub>; 500 µg of D-Cloprostenol, Ciclase DL<sup>®</sup>, Zoetis, Argentina) to induce luteolysis and intravaginal device was removed.

Ovaries of each llama were monitored using ultrasonography from Day 0 to 7 and again on day of embryo collection. Females with less than three follicles  $\geq 7$  mm on Day 7 were considered non-responders (Huanca et al., 2009); therefore, were not mated and were not included in the study. At Day 7 following estrus, females were naturally mated with males of proven fertility and treated with 100 µg of GnRH analogue as an additional stimulus to induce ovulation. A second mating was allowed 24 h later.

Embryo recovery from the donor females was performed non-surgically on Days 14 or 15 (7 or 8 days after the first mating) as described previously (Aller et al., 2010). Briefly, each female was sedated with 10 mg acepromazine maleate i.m. (Acedan<sup>®</sup>, Holliday, Argentina) and then was placed in sternal recumbent position on a table to facilitate the procedure. Caudal epidural anesthesia with 3 mL of 2% lidocaine hydrochloride was induced before uterine flushing. Each uterine horn was flushed using a 14-Fr Rusch two-way catheter and 250 mL of Ringer lactate supplemented with 2% heat-inactivated calf serum and antibiotics. The flushing medium was filtered (EmCon, Minitüb, Germany) and searching embryos was performed using stereomicroscope at magnification 40 $\times$ . The recovered embryos were transferred to holding medium (Syngro Holding Medium<sup>®</sup>, Bioniche Animal Health, Canada) and maintained at room temperature and classified according to the visual appearance of the surrounding trophectoderm cells (Grade 1 = excellent, Grade 2 = moderate to good, Grade 3 = poor and Grade 4 = holed) (Vaughan et al., 2013; Aller et al., 2015a). The ovaries of each llama were monitored by ultrasonography immediately after embryo collection. The number of CL and the number of large (> 7 mm) follicles from which ovulation had not occurred were recorded.

### 2.3. Blood collection and hormone determinations

A single blood samples was collected from Day 0 to 9. One more sample was collected on day of embryo collection. Each sample was collected by jugular venipuncture into tubes containing sodium heparin (Fada Pharma, Argentina) and immediately placed on ice for as long as 2 h until centrifugation at 1500 g for 20 min. Plasma was then stored at  $-20^{\circ}\text{C}$  until hormone analyses were conducted. Concentrations of P<sub>4</sub> in plasma samples were determined using a radioimmunoassay (RIA) commercial kit (Diagnostics Products Corporation; Los Angeles, CA, USA) for use in cattle and validated for llama (Bianchi et al., 2007). The intra-assay coefficient of variation was 5% for concentrations between 0.10 and 40.0 ng/mL. The estimated sensitivity of this method was 0.01 ng/mL. Concentrations of E<sub>2</sub> were determined on Days 6, 7, 8 and 9 using a RIA commercial kit (Diagnostics Products Corporation; Los Angeles, CA, USA) for use in cattle and validated for use with llama plasma after minor modifications (Aba et al., 1995). Intra-assay coefficient of variation was < 16% for concentrations between 5.6 and 180 pmol/L. The estimated assay sensitivity using this method was 5.6 pmol/L. Concentrations of all samples were determined in duplicates and in a single assay for each hormone.

### 2.4. Statistical analyses

Data analyses were performed using SAS Institute Inc. (1989). Number of follicles  $\geq 7$  mm, number of CL and number of recovered embryos were compared between groups using one-way ANOVA. Plasma P<sub>4</sub> and E<sub>2</sub> concentrations were analyzed using an ANOVA for repeated measures (PROC MIXED); the statistical model included the effects of treatment, day of treatment and treatment by day interaction, with day as a repeated effect. Female within treatment was included as a random effect. Proportional data were compared between groups by Fisher's exact test. Pearson correlation coefficients were also conducted to evaluate the relationship among steroid hormone (E<sub>2</sub> and P<sub>4</sub>) concentrations and number of follicles on day of mating, number of CL and number of recovered embryos (CORR procedure). Correlation coefficients were classified as strong ( $r > 0.6$ ), moderate ( $r = 0.4 - 0.6$ ) or weak ( $r < 0.4$ ). Data are presented as mean  $\pm$  SD and probabilities of < 5% ( $P < 0.05$ ) were considered to be statistically significant.

## 3. Results

### 3.1. Superovulatory response of follicles and embryo yield

There were 51 of 54 (94.4%) females that had the superovulation treatment regimen imposed with mature follicles (at least three follicles  $\geq 7$  mm per female) detected by ultrasonography on day of mating. There were two of 27 (7.4%) llamas from the P<sub>4</sub> group and 1 of 27 (3.7%) in the control group that had only one follicle  $\geq 7$  mm on Day 7 and these animals were not mated and were not included in the study. The rest of the females were sexually receptive and successfully mated.

The mean number of follicles  $\geq 7$  mm in diameter at the time of mating was greater ( $P = 0.04$ ) in non-treated llamas than in the P<sub>4</sub>-treated llamas. Similarly, mean number of follicles  $\geq 7$  mm (follicles from which there was no ovulation) on day of embryo recovery was greater ( $P < 0.01$ ) in the control group than P<sub>4</sub>-treated group (Table 1). The mean number of CL on the day of embryo recovery and the mean number of embryos recovered were similar ( $P > 0.05$ ) for each treatment group (Table 1). A total 106 embryos were recovered from 51 llamas in which uterine flushing occurred (2.1 embryos per female) and all recovered embryos were at the hatched blastocysts stage and were graded to be of excellent ( $n = 77$ , 72.6%) and moderate to good ( $n = 29$ , 27.4%) quality (Fig. 1). There were no Grade 3 and 4 embryos recovered. The number of recovered embryos per uterine flushing regimen ranged from zero to seven and the number of llamas producing multiple embryos is depicted in Fig. 2. There were 35 of 51 (68.6%) llamas that produced at least one embryo which indicates that approximately a third (31.4%) of the animals did not respond to the

**Table 1**

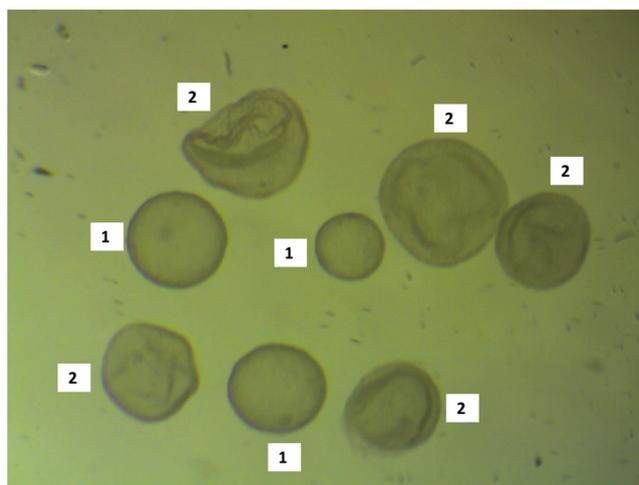
Ovarian response and embryo recovery (mean  $\pm$  SD) in llamas not treated (Control group) or treated with a 5-day intravaginal device (DIB 0.5%; P<sub>4</sub> group) and superstimulated for follicular development with eCG at the time of follicular wave emergence 2 days after induced ovulation with a GnRH analogue.

Item	Treatment		
	P <sub>4</sub> group (n = 25)	Control group (n = 26)	P
Follicles $\geq$ 7 mm on day of mating (range)	6.7 $\pm$ 4.9 (0–18)	9.7 $\pm$ 4.2 (0–17)	0.04
Follicles > 7 mm on day of embryo recovery (range)	1.6 $\pm$ 1.5 (0–6)	4.5 $\pm$ 2.7 (0–13)	< 0.01
CL on day of embryo recovery (range)	5.2 $\pm$ 3.4 (0–12)	5.5 $\pm$ 3.1 (0–10)	0.99
Ovulation rate (%) <sup>a</sup>	130/168 (77.4)	135/253 (53.3)	< 0.01
Ovulation incidence (%) <sup>b</sup>	23/25 (92.0)	25/26 (96.1)	0.60
Embryo recovery rate (%) <sup>c</sup>	51/130 (39.2)	55/135 (40.7)	0.90
Embryos recovered (range)	2.0 $\pm$ 1.8 (0–7)	2.1 $\pm$ 2.2 (0–7)	0.94
Embryo Grade 1 (excellent, %)	42/51 (82.4)	36/55 (65.4)	0.07
Embryo Grade 2 (moderate to good, n)	9/51 (17.6)	19/55 (34.6)	0.07

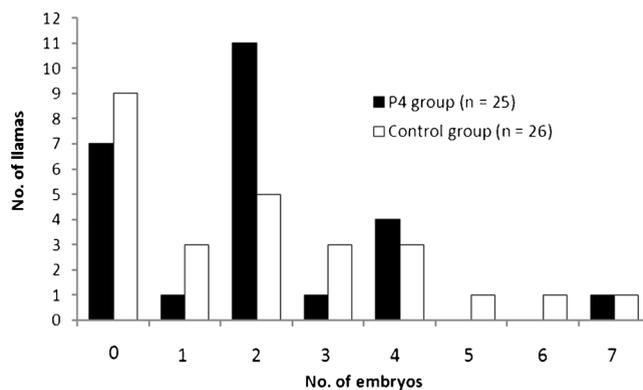
<sup>a</sup> No. of CL on day embryo recovery/no. follicles on day of mating.

<sup>b</sup> No. of ovulated/no. treated females.

<sup>c</sup> No. of recovered embryos/no. of CL on day of embryo recovery.



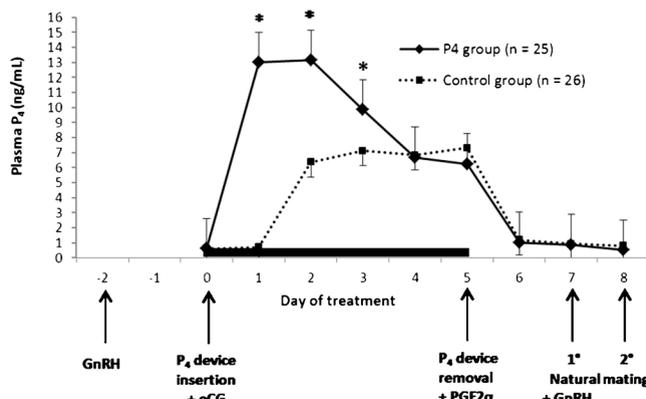
**Fig. 1.** Grade 1 (excellent) and Grade 2 (moderate to good) llama embryos recovered at Day 7 after natural mating.



**Fig. 2.** Number of llamas producing from 0 to 7 embryos.

superovulatory treatment by producing embryos (7/25 = 28.0% and 9/26 = 34.6% for P<sub>4</sub> and control groups, respectively,  $P = 0.76$ ). The proportion of excellent embryos (grade 1) tended to be greater ( $P = 0.07$ ) in the P<sub>4</sub> group than the control group (Table 1).

There were positive correlations between number of follicles  $\geq$  7 mm on day of mating and number of CL on day of embryo collection (P<sub>4</sub> group;  $r = 0.65$ ,  $P = 0.002$  and control group;  $r = 0.75$ ,  $P = 0.001$ ). There, however, were no significant correlations



**Fig. 3.** Mean ( ± SD) plasma progesterone (P<sub>4</sub>) concentrations in llamas not treated (Control group) or treated with a 5-day intravaginal device (DIB 0.5°; P<sub>4</sub> group) and superstimulated with eCG at the time of follicular wave emergence 2 days after induced ovulation with aGnRH analogue; Solid bar indicates when intravaginal device was *in situ*; (\*) Mean values differ between groups at *P* < 0.05.

between number of CL and number of embryos (P<sub>4</sub> group; *r* = 0.25, *P* = 0.30 and control group; *r* = 0.27, *P* = 0.24).

### 3.2. Plasma P<sub>4</sub> and E<sub>2</sub> concentrations

There was a day of treatment by treatment interaction on P<sub>4</sub> concentrations (*P* < 0.01). Animals administered P<sub>4</sub> had greater (*P* < 0.05) plasma P<sub>4</sub> concentrations from Day 1 to Day 3 than animals of control group with an average ( ± SD) peak concentration of 13.0 ± 1.9 ng/mL (Day 2) and 7.2 ± 1.2 ng/mL (Day 5), respectively. From Day 4 to 8, there were no differences (*P* > 0.05) in plasma P<sub>4</sub> concentrations between groups (Fig. 3).

Plasma P<sub>4</sub> concentrations (mean ± SD) on day of embryo collection were not different (*P* > 0.05) between groups (P<sub>4</sub> group = 33.8 ± 11.3 ng/mL and control group = 33.5 ± 10.7 ng/mL). In addition, plasma P<sub>4</sub> concentrations (mean ± SD) in llamas that produced ≥ two embryos (33.7 ± 12.1 and 34.0 ± 8.7 ng/mL) were not different (*P* > 0.05) from those of llamas producing one or no embryos (31.1 ± 10.1 and 32.6 ± 14.7 ng/ml) for P<sub>4</sub>-treated and control groups respectively. Data for correlations between plasma P<sub>4</sub> and E<sub>2</sub> concentrations and number of follicles on day of mating, number of CL and number of embryos on day of embryo collection are included in Table 2.

There was a day by treatment interaction (*P* < 0.01) on plasma E<sub>2</sub> concentrations. Plasma E<sub>2</sub> concentrations determined on the previous day (Day 6) and on day of the first mating (Day 7) were greater (*P* < 0.05) in llamas of the control than those of the P<sub>4</sub>-treated group. In the 2days after the first mating (Day 8 and Day 9) there were no differences (*P* > 0.05) in plasma E<sub>2</sub> concentrations between groups (Fig. 4).

## 4. Discussion

The results of the present study indicate that llamas in which there was imposed superstimulation of follicular development with eCG administered at the time of follicular wave emergence in the early luteal phase and without supplementation with exogenous P<sub>4</sub> had a greater ovarian response as determined by number of follicles ≥ 7 mm at the time of mating than in P<sub>4</sub>-treated llamas. Llamas treated with a P<sub>4</sub>-intravaginal device at the time of administration of eCG for ovarian follicular superstimulation had greater plasma P<sub>4</sub> concentrations the first 3 days of treatment and had similar numbers of CL and recovered embryos of llamas without P<sub>4</sub>-treatment. These findings are consistent with the results reported by Huanca et al. (2009), where it was observed that imposing a superstimulatory regimen for follicle development using eCG alone administered at time of emergence of the follicular wave 2 days after

**Table 2**

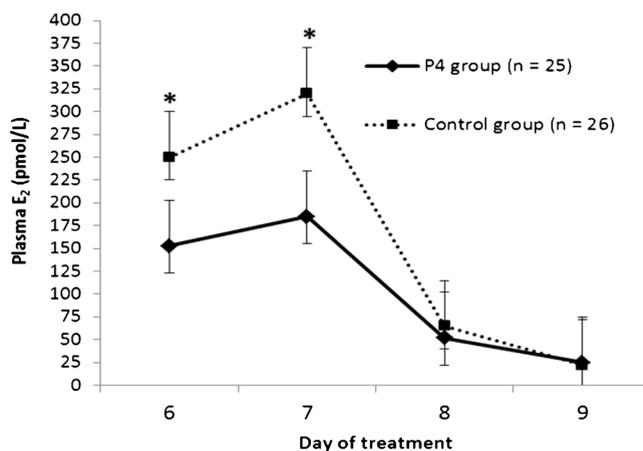
Correlation coefficients between the numbers of follicles on day of mating, corpora lutea and recovered embryos and the peak values of E<sub>2</sub> during the peri-ovulatory period and P<sub>4</sub> concentrations on day of embryo recovery.

	E <sub>2</sub> (peak)		P <sub>4</sub> (day of embryo collection)	
	P <sub>4</sub> group	Control group	P <sub>4</sub> group	Control group
Follicles (on day of mating)	0.98**	0.91**	0.52*	0.45*
CL (day of embryo recovery)	0.46*	0.57*	0.59**	0.53*
Recovered embryos	0.28	0.10	0.37	0.14

P<sub>4</sub> group, *n* = 25; Control group, *n* = 26.

\* *P* < 0.05.

\*\* *P* < 0.01.



**Fig. 4.** Mean ( $\pm$  SD) plasma estradiol-17 $\beta$  ( $E_2$ ) concentrations of llamas treated for superovulation; Day 7 = first natural mating; Day 8 = second natural mating; (\*) Mean values differ between groups at  $P < 0.05$ .

LH administration resulted in a greater number of follicles  $> 7$  mm in diameter at the time of mating than when there was with an eCG + MPA (intravaginal sponge impregnated with medroxyprogesterone acetate) treatment regimen.

The use of  $P_4$  (intravaginal device) to synchronize the time of follicular wave emergence obviates the need to know the specific follicular stage when starting the superovulatory treatment and this treatment should be initiated near the time of follicular wave emergence to result in the maximal superovulatory response (Adams et al., 1994). In the current study, the inclusion of a  $P_4$ -intravaginal device had an inhibitory effect on the total number of follicles at the time of mating (Day 7); however, the numbers of CL and recovered embryos were not different with use of the two protocols. By contrast, Aba et al. (2005) reported that the use of an intravaginal progesterone device (CIDR 0.33 g) in vicuñas resulted in inhibition of the development of a dominant follicle, but when this device was used in combination with eCG for follicular superstimulation there was an enhanced ovarian response. By contrast, when large doses of  $P_4$  (600 mg/day i.m.) were used in FSH-treated cows, a greater population of follicles  $\geq 10$  mm developed after superovulatory treatment for follicular development (Callejas et al., 2008).

In sheep, the presence of a CL at the beginning of FSH treatment did not have a significant effect on the growth of follicles in response to the gonadotropin treatment, or on the number of ovulations (Veiga-Lopez et al., 2005). Similarly, in the present study, llamas without supplementation of exogenous  $P_4$  and having a functional CL had sufficient endogenous  $P_4$  concentrations to allow the development of a large number of follicles  $\geq 7$  mm at the time of mating. In contrast, llamas treated with a  $P_4$ -intravaginal device had relatively greater plasma  $P_4$  concentrations the first 3 days of treatment, therefore, the emergence and development of new follicles was partially inhibited by the treatment.

In the present study, relatively greater plasma  $E_2$  concentrations in the peri-ovulatory period in animals of the control group resulted in the failure of ovulation from some developing follicles; consequently, the ovulation rate of the control group (53.3%) was less than the  $P_4$ -group (77.4%). Although, the number of pre-ovulatory follicles  $\geq 7$  mm was greater in llamas of the control than MPA-treated group, the ovulatory rates were similar between treatment groups (Huanca et al., 2009). In the present study, the embryo recovery rates (number of recovered embryos/number of CL) were similar between treatment groups and are consistent with results where there was a very large data set (Vaughan et al., 2013) from commercial alpaca embryo transfer records (41.4%) and llamas (44.8%; Huanca et al. (2009). Nevertheless, Correa et al. (1997) and Ratto et al. (1997) reported lesser embryo recovery rates (34.5% and 16.9%, respectively). Embryo recovery rate was just 22% in a study with lactating llamas (Aller et al., 2010).

In llamas and other domestic species, embryo yield is still highly variable and unpredictable. The mean number of recovered embryos per collection in the present study was similar to those observed in other previous studies in llamas (Bourke et al., 1995; Correa et al., 1997; Aller et al., 2002, 2010) and alpacas (Vaughan et al., 2013). When eCG alone and eCG + MPA protocols were used a greater mean number of embryos per llama (4.8 and 3.5, respectively) were recovered (Huanca et al., 2009).

In the present study, the recovery rate of embryos with an excellent (grade 1) grade obtained from  $P_4$ -treated llamas (82.4%) was greater (although not statistically significant possibly because of too few llamas per treatment group) than that of non-treated llamas (65.4%). As an alternative explanation for reduced embryo quality in control group, the greater number of follicles ( $\geq 7$  mm) observed on Day 7 (time of mating) resulted in greater plasma  $E_2$  concentrations, that might have resulted in a decreased secretory activity of the oviductal epithelial cells (Van de Leemput et al., 1999) and change the oviductal milieu impairing fertilization and subsequent embryo development (Callesen et al., 1986). In a recent study with sheep, Menchaca et al. (2018) reported that although the follicular population was not affected by the  $P_4$  treatment, oocyte quality, cleavage rate and *in vitro* embryo development were significantly improved, due to greater oocyte competence.

In cattle, it has been reported that superstimulation of follicular development when there are less luteal phase  $P_4$  concentrations there was a reduced embryo quality (Goto et al., 1988). A positive effect of greater plasma  $P_4$  concentrations at the beginning of the superovulatory treatment on embryo quality was observed in sheep (Gonzalez-Bulnes et al., 2002; Cuadro et al., 2018; Menchaca et al., 2018) and cattle (Nasser et al., 2011; Rivera et al., 2011). Results of these studies indicate supplementation with exogenous  $P_4$

during superstimulatory follicular treatment initiated at the time of emergence of the first follicular wave after ovulation (low endogenous P<sub>4</sub>) improved the embryo yield, mainly by increasing the fertilization rate and ova/embryo quality. The hormonal treatment performed in the present study in llamas, could be considered similar to those protocols reported in sheep and cattle because eCG was administered at the time of emergence of the second follicular wave of the estrous cycle after GnRH injection to induce ovulation.

In cattle, there is modulation of follicular development as compared to that of typically developing follicles during the estrous cycle as a result of imposing superovulatory protocols with gonadotropins, especially when eCG is used (Yadav et al., 1986). This leads to the ovulation of oocytes with abnormalities that compromise developmental competence, fertility and alter the normal processes of early embryo development (Greve et al., 1995). In addition, the relatively long half-life of eCG compared with most hormones also results in a protracted ovarian stimulation, follicles from which there is not ovulation, abnormal endocrine profiles and reduced embryo quality (Henricks et al., 1973; Mikel-Jenson et al., 1982). The results of the present study and in other species, therefore, indicate there is a clear association of relatively lesser P<sub>4</sub> concentration during superstimulatory follicular treatments (i.e., during at least 3 days before ovulation (follicular recruitment) and a large number of developing follicles, a greater E<sub>2</sub> concentration at the time of mating, lesser ovulation rate and compromised subsequent embryo development. At present, the precise effect of relatively greater P<sub>4</sub> concentrations during follicular wave development on the oocyte remains to be elucidated (Fair and Lonergan, 2012).

In the present study, there was a positive association between E<sub>2</sub> peak concentration and the mean number of follicles  $\geq 7$  mm on day of mating and mean numbers of CL and recovered embryos and these findings are consistent with results from previous research in cattle (Goto et al., 1988). There, however, was no significant correlation between E<sub>2</sub> peak concentration and number of embryos per llama in the present study. There was a positive correlation between the mean number of CL per llama and plasma P<sub>4</sub> concentration in the present study and this finding is consistent with that in a previous study with sheep (Samartzi et al., 1995) and llamas (Bourke et al., 1995). There was, however, no significant association between mean number of CL and mean number recovered embryos for both treatment groups which is similar to the findings of Aller et al. (2010), possibly as consequence of a large number of follicles at the time of mating; therefore, the ovarian bursa can be displaced leading to a loss of oocyte into the abdominal cavity and a lesser number of recovered embryos, but it remains unknown whether this occurs in llamas.

## 5. Conclusion

Results of the present study indicate administration of eCG for superovulation during the period when there was a natural early luteal phase occurring without treatment with exogenous P<sub>4</sub> induced a new wave of follicle development after ovulation that led to an abnormal peri-ovulatory hormonal balance with a large number of follicles and greater E<sub>2</sub> concentrations, which may be detrimental to early embryonic development. This superovulatory treatment may disrupt normal oocyte and follicular development leading, ultimately, to embryos of inferior quality.

When gonadotropin treatment is initiated at the time of follicular wave emergence in the early luteal phase it will be necessary to supplement with exogenous P<sub>4</sub> to partially inhibit follicular development, decrease plasma E<sub>2</sub> concentrations and improve embryo quality in llamas. Results also supported the hypothesis that P<sub>4</sub> concentrations during superovulatory treatment and E<sub>2</sub> concentrations during the peri-ovulatory period are related to ovarian response, ovulation rate and embryo quality.

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