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SHORT COMMUNICATION

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Embryo yield in llamas synchronized with two different intravaginal progesterone-releasing devices and superovulated with eCG

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Abstract

The objectives of this study were to compare the effects of two intravaginal devices (ID) containing the same dose (0.5 g) of progesterone (P_4) on subsequent ovarian response, embryo production and circulating P_4 concentration profile in llamas (*Lama glama*) treated with equine chorionic gonadotropin (eCG) for ovarian superstimulation. Female llamas were randomly assigned (n = 10 llamas per group) to one of the following groups and treated (Day 0) with an ID containing 0.5 g of vegetal P_4 to synchronize the emergence of a new follicular wave: i) DIB 0.5® and ii) Cronipres M15®. On Day 3 llamas were intramuscularly treated with 1000 IU of eCG. The IDs were removed on Day 7. Llamas were naturally mated (Day 9) and treated with GnRH analogue to induce ovulation. A second mating was allowed 24 h later. Embryos were collected between 7 and 8 days after the first mating. Blood samples were taken every day from Day 0 to Day 7 to measure circulating P_4 concentrations. The results indicated that DIB device maintained greater plasma P_4 levels as compared to Cronipres until Day 2. However, the mean (± SD) number of corpora lutea and recovered embryos was not affected ($p < 0.05$) by the type of ID (5.3 ± 2.6 *vs* 4.2 ± 2.2 and 3.5 ± 2.7 *vs* 2.6 ± 3.0 for DIB and Cronipres, respectively). In conclusion, both DIB and Cronipres devices can be successfully used to synchronize the emergence of follicular wave prior to a single dose of eCG in superovulation protocol in llamas.

Additional key words: South American camelids; superovulation; hormonal treatment; embryo.

Abbreviations used: CL (corpora lutea); eCG (equine chorionic gonadotropin), GnRH (gonadotropin-releasing hormone); ID (intravaginal device); LH (luteinising hormone); MOET (multiple ovulation and embryo transfer); P_4 (progesterone); SAC (South American camelids).

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The South American camelids (SAC), also known as the New World Camelidae, are mainly reared in the High Andes having an important economic and social role in countries such as Argentina, Bolivia, Chile, Peru and in recent years, Ecuador. Eighty-six per cent of the world´s 3.5 million alpacas are found in Peru; likewise, the greatest percentage (>70%) of the world´s 3.1 million llamas are found in Bolivia (Novoa, 1981). The greater population of guanacos is found in the Argentinean Patagonia, with an estimated number of 600,000 animals and the vicuña population is distributed among all the aforementioned countries. In those countries, several programs for genetic improvement (meat and fibre) have been undertaken based on assisted reproductive techniques.

Multiple ovulation and embryo transfer (MOET) allow the decrease of generational interval, optimization of mating programs and increasing the reproductive potential from high genetic value females. In llama and alpaca, the MOET is more used than artificial insemination due to poor results using frozen semen (Bravo *et al*., 2013). Ovarian superstimulation in SAC is performed under different general conditions: i) simulating a luteal phase by using exogenous proges-

terone/progestogen, ii) during a luteal phase following ovulation induced by administration of hCG (human chorionic gonadotropin), LH (luteinising hormone), GnRH (gonadotropin-releasing hormone) or iii) after ablation of all follicles greater than 5 mm using transvaginal ultrasound-guided follicle aspiration. The simplest method to control follicular dynamics and synchronization of the emergence of a new follicular development in llama is to use an intravaginal device (ID) (Chaves *et al*., 2002) or an ear implant containing progestogen (Bourke *et al*., 1995).

Progesterone may also have an important effect on oocyte quality decreasing the apoptosis rate of cumulus cells during maturation (Salhab *et al.,* 2011). On the other hand, a positive relationship between greater circulating levels of P_4 during follicular growth and embryo quality have been recently described in dairy cows (Rivera *et al*., 2011). The precise effect of elevated P_4 during the growth of the dominant follicle on the oocyte and embryo quality is unknown (Fair & Lonergan, 2012) but it may be related to reduced exposure of the developing oocyte to LH, avoiding its premature maturation (Cerri *et al*., 2011).

In Argentina, different kinds of IDs containing different amounts of P_4 for use in cattle have been developed by pharmaceutical industry in the last 10 years and are exported to all South America, especially to the Andean countries where the SAC are raised. Moreover, IDs containing low dose ($\leq$ 0.5 g) of P_4 should preferably be used to reduce environmental pollution after use in animals.

These IDs, that contain the same P_4 dose, vary in shape and surface contact area, variations which could have an effect on the synchronization obtained. Thus, differences in physical characteristics among IDs produce different plasma P_4 concentrations in cattle (Rathbone *et al*., 1998; 2002). In a recent study, van Werven *et al.* (2013) found that PRID® produced significantly greater circulating P_4 concentration compared to CIDR® and suggested that both levels of P_4 content and differences in shape and contact surface area might contribute to circulating P_4 concentrations.

Therefore, the objectives of the present work were to compare the effects of two commercially available and commonly used IDs containing low dose of P_4 and varying in their structure and shape on subsequent bioavalability in blood plasma, ovarian response and embryo yield in llamas treated with eCG for ovarian superstimulation.

This work was carried out at Abra Pampa Experimental Station of the National Institute of Agricultural Technology (INTA), located in the province of Jujuy (22º 49´S, 65º 47´W; 3,483 m above sea level) during non-breeding season. Animals weighed 102.3 ± 7.8 kg, with a body condition score of 4.6 ± 1.1 (1 = emaciated to 9 = obese; Richards *et al*., 1986) determined by palpation. Llamas were selected (n = 20 from a group of 60) and randomly allocated in equal numbers (n = 10) to one of two groups to be treated (Day 0) with an intravaginal device (i) DIB 0.5® (Syntex, Argentina) (DIB group) or (ii) Cronipres M15® (Biogenesis-Bago, Argentina) (Cronipres group) and both IDs containing 0.5 g of progesterone. Each ID was removed seven days later. Ovarian follicular development was stimulated by intramuscular administration of 1000 IU of eCG (Novormon 5000®, Syntex, Argentina) (Day 3). Llamas were naturally mated (Day 9) with males of proven fertility and immediately treated with 100 µg of GnRH analogue of gonadorelin (Gonasyn GDR®, Syntex, Argentina) 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 7 days after the first mating as described previously (Aller *et al*., 2002). Briefly, each female was sedated with 10 mg acepromazine (Acedan®, Holliday, Argentina) and caudal epidural anaesthesia with 3 mL of 2% lidocaine hydrochloride was induced before uterine flushing. Each uterine horn was flushed using 14-Fr Rusch two-way catheter and 250 mL of Ringer lactate supplemented with 1% heat-inactivated cow serum. The flushing medium was filtered (EmCon, Minitüb, Germany) and searching embryos was performed using a stereomicroscope at magnification x40. The recovered embryos were transferred to holding medium (Syngro holding medium®, Bioniche Animal Health, Canada) and kept at room temperature and classified according to IETS standards for cattle embryos (IETS Manual, 1998). After embryo collection, ovarian responses in the donor females were evaluated by transrectal ultrasonography using a real time scanner with a 5-MHz linear array transducer (Honda HS 101V, Japan).

Blood samples were collected on Days 0, 1, 2, 3, 4, 7 and 9 by jugular venipuncture into tubes containing sodium heparin (Fada Pharma, Argentina) and immediately centrifuged at 1500 x*g* for 20 min. The blood plasma was stored at –20ºC until hormone analyses. Concentrations of P_4 were measured by a radioimmunoassay commercial kit (Siemens Medical Solutions Diagnostics, Los Angeles, CA, USA) for use in bovine and validated for llama (Aba *et al.*, 1999). Samples were evaluated in duplicate and all samples were analyzed in a single assay. The intra- assay coefficient of variation was < 9%, for concentrations between 0.1 and 40.0 ng/mL. The estimated sensitivity of this method was 0.01 ng/mL.

Data analyses were performed using SAS (1989). Number of unovulated follicles ≥ 7 mm, number of

corpora lutea (CL) and number of recovered embryos were compared between groups using one-way ANOVA. Plasma P_4 concentration was analyzed by ANOVA for repeated measures (PROC MIXED); the model included the effects of treatment, day and treatment by day interaction, with day as a repeated effect. Proportional data were compared by Chi square test. Correlation coefficients (Pearson correlation) between number of CL and number of recovered embryos were calculated (Steel & Torrie, 1980). Probability of $p < 0.05$ was considered to be statistically significant.

On Day 9, all llamas were sexually receptive and successfully mated. After device removal, a slight vaginitis was observed; however, the fertility was not compromised because ejaculation is deep intracornual in this species. The mean number of unovulated follicles ≥ 7 mm, CL and recovered embryos were not affected ($p > 0.05$) by treatment (Table 1). The recovery rates (total number of recovered embryos relative to the total number of CL) were 66.0 (35/53) and 61.9% (26/42) for DIB and Cronipres groups respectively ($p > 0.05$). All recovered embryos, regardless of group, were graded as excellent quality hatched blastocysts. Embryos could not be collected from 4 of the 20 animals (two animals of each group). Approximately 20% of the animals did not respond to the superovulatory treatment. Significant positive correlations between number of CL and number of recovered embryos for DIB and Cronipres groups were detected ($r = 0.68$, $p = 0.02$ and $r = 0.80$, $p = 0.005$, respectively).

The circulating P_4 concentrations of females from the DIB and Cronipres groups are shown in Fig. 1. An effect of Day × Treatment interaction was detected on plasma P_4 concentrations. Significant differences ($p < 0.05$) in P_4 concentration between groups were determined at 24 and 48 h after ID insertion; however these differences were not observed from Day 3 onwards. Plasma P_4 concentration (mean ± SD) in llamas that yielded ≥ 2 embryos in both Groups (DIB = 35.6 ± 19.0 ng/mL; Cronipres = 32.0 ± 10.6 ng/mL) were not different ($p < 0.05$) from those in llamas yielding single or no embryo (DIB = 28.8 ± 16.8 ng/mL; Cronipres = 22.6 ± 14.2 ng/mL).

The main finding from this experiment was that both intravaginal devices (DIB and Cronipres) containing low dose of progesterone used during superovulation treatment produced a similar ovarian response and number of recovered embryos in llamas.

Several research groups have looked for possible associations between endogenous (natural luteal phase) or exogenous progesterone priming (treatment used to control the ovarian follicular dynamics) and the response to superovulation as a potential explanation for some of the persistent variability in superovulation seen between animals (Kanitz *et al*., 2002; Mapletoft *et al*., 2002). In this study, the aim was to investigate any differences in quantity and quality of embryos produced in response to either DIB or Cronipres progesterone ID combined with eCG for ovarian superstimulation in llamas.

The use of progesterone to synchronize the follicular wave emergence obviates the need to know the specific follicular stage when starting the superovula-

Table 1. Ovarian response and embryo yield (mean ± SD; range in parenthesis) in llamas treated with 1000 IU of eCG (equine chorionic gonadotropin) after synchronization of follicular wave emergence with two different intravaginal devices containing 0.5 g of progesterone

	DIB Group (n = 10) [1]	Cronipres Group (n = 10) [1]	*p*
No. of follicles ≥ 7 mm on day of embryo recovery	2.5 ± 2.7 (0-9)	3.3 ± 3.2 (0-8)	0.55
No. of CL [2] on day of embryo recovery	5.3 ± 2.6 (3-11)	4.2 ± 2.2 (3-10)	0.31
No. of recovered embryos	3.5 ± 2.7 (0-8)	2.6 ± 3.0 (0-10)	0.48

[1] Two females of each group did not respond to the superovulatory treatment (0 embryo). [2] CL: corpora lutea.

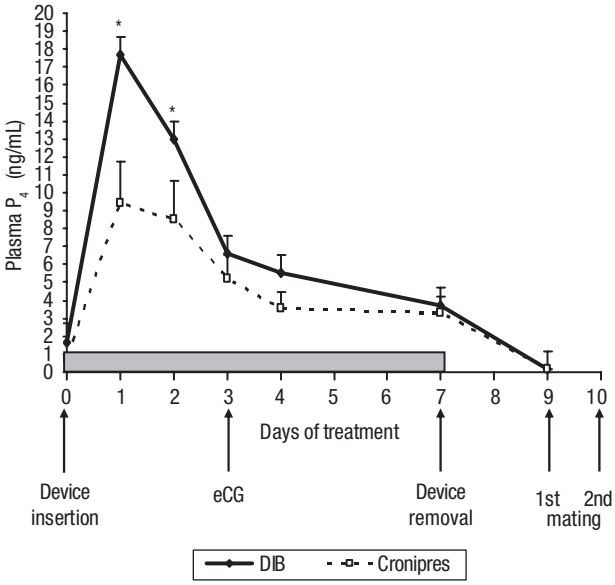

Figure 1. Mean (± SD) concentrations of P_4 in plasma of llamas treated with an intravaginal device containing 0.5 g of progesterone (DIB or Cronipres) during 7 days and 1000 IU eCG for superovulation. * DIB differed from Cronipres on Day 1 and Day 2 ($p < 0.05$). P_4: progesterone; eCG: equine chorionic gonadotropin.

tory treatment and this treatment should be initiated near the time of follicular wave emergence to produce the maximal superovulatory response (Adams *et al*., 1994). In llamas, Aller *et al*. (2010) observed that the follicular wave emergence occurred approximately on Day 4 (± one day) after medroxiprogesterone acetate intravaginal sponge insertion. Therefore, in the present study the eCG treatment for ovarian stimulation was administered on Day 3 after ID insertion.

Superovulatory treatment can be initiated in natural luteal phase, but require the use of ultrasonography to detect the growing dominant follicle and then to induce ovulation with human chorionic gonadotrophin (San Martin *et al*., 1968) or luteinizing hormone (Fernandez-Baca *et al*., 1970). Therefore, we chose exogenous progesterone because this hormone allows regulate the ovarian function indirectly through LH secretion, rather than by direct actions on the ovaries. The P_4 device is very simple to apply in field conditions to control the ovarian follicular dynamics and predicts the emergence of a new follicular wave and thus to start the ovarian superstimulatory treatment without the use of ultrasound.

In the present study the number of CL and embryos recovered per llama did not differ between DIB and Cronipres group. Huanca *et al*. (2009) using progestin-realising vaginal sponges obtained a greater number of CL (8.6) but the number of recovered embryos (3.5) was similar to our study. Additionally, Carretero *et al*. (2010), who used daily intramuscular administration of progesterone during five days to inhibit follicular growth obtained 2.9 embryos per female. Progestogen implants inserted over a period of 7 days combined with 1000 IU of eCG on Day 5 yielded a low embryo recovery (1.3 embryos per donor female); however, the same protocol using CIDR® improved the embryo recovery (2.0 embryos) (Bourke *et al*., 1992).

The recovery rates for DIB and Cronipres groups were significantly higher than the recovery rates obtained from other small-scale studies in llama (Correa *et al*., 1997, 34.5%; Ratto *et al*., 1997, 16.9%). On the other hand, in a very large data set collected recently by Vaughan *et al*. (2013) from commercial alpaca embryo transfer records the recovery rate was 38.8% (4188 embryos/10796 ovulations).

The effect of elevated progesterone during follicular growth has been linked to improved embryo quality (Lonergan, 2011). Additionally, Rivera *et al*. (2011) showed that high P_4 during ovarian superstimulation treatment of lactating dairy cows increased the quality of embryos collected on Day 7 after estrus. In the present study, all recovered embryos were hatched blastocysts graded as excellent quality. The associations between number of CL and recovered embryos for DIB and Cronipres groups were similar to that observed by Vaughan *et al*. (2013) in alpacas ($r = 0.54$). However, no significant correlation ($r = 0.12$) was detected by Aller *et al*. (2010), possibly as consequence of the high number of ovarian follicles (12.4) induced by the superovulatory treatment; therefore, the ovarian bursa can be displaced leading to a loss of oocytes into the abdominal cavity and a low number of recovered embryos.

Plasma P_4 concentrations rose quickly after ID insertion (Day 0) with peak concentration attained on Day 1. Significant differences of circulating P_4 concentrations between DIB and Cronipres devices were observed at this day. Following 48 h after device removal, P_4 concentrations were not significantly different to pre-treatment concentrations. Treatments with an intravaginal CIDR® device containing 0.33 g of progesterone (Chaves *et al*., 2002) and medroxiprogesterone acetate-vaginal sponge (Aba *et al*., 1999; Huanca *et al*., 2009) were successfully used to control ovarian follicular dynamics. Progesterone concentrations similar to the one observed in our study was described by Chaves *et al*. (2002) where a rapid increase was observed at Day 1 (~10 ng/mL) and sharply decreased until Day 4 (2 ng/mL).

The two types of ID used in the present study have the same P_4 content (0.5 g), therefore differences in contact surface area (DIB ~95 cm^2 *vs* Cronipres ~60 cm^2) might contribute to differences in plasma P_4 concentrations. Additionally, because the shape of the two IDs differ greatly (DIB = V-shape *vs*. Cronipres = double L-like with three hood containing progesterone) it may influencing the surface area of the devices in direct contact with the vagina wall since the rate of diffusion of P_4 from ID to bloodstream could be considered similar, because the two IDs have the same type of outer layer material (inert silicone).

The results supported the hypothesis that the shape and contact surface area with the vagina wall of two ID (DIB and Cronipres) containing the same P_4 levels have effect on the plasma P_4 concentrations. In spite of the differences in physical characteristic and the plasma P_4 concentrations observed between devices, the ovarian superstimulation protocols using eCG combined with DIB or Cronipres were equally effective to induce a good superovulatory response and embryo production.

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