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Embryo production in superstimulated llamas pre-treated to inhibit follicular growth

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ABSTRACT

Llamas are monotocous and the length of their gestation period varies between 342 and 350 days. Thus the average number of offspring any female can produce throughout her reproductive life is very limited to spread a desired genome. The multiple ovulation and embryo transfer (MOET) technique allows an alternative to this limitation and reduces the generation interval. The objective of this study was to evaluate embryo recovery in superstimulated llamas which had previously been hormone-treated to inhibit follicular growth. A total of 50 female llamas were monitored daily via rectal palpation and ultrasound and divided according to their ovarian follicular growth into four phases. The females in each phase were then randomly divided into two groups: *A* ($n = 20$) received a single dose of 1 mg of estradiol benzoate (EB) on the first day of the treatment + 100 mg of progesterone (P4) i.m. for 5 days with 5 animals per phase and *B* ($n = 20$) received 1 mg EB at onset + 150 mg P4 i.m. for a period of 5 days with 5 animals per phase. Group *C* ($n = 10$) or control did not receive any prior hormonal treatment and the females were in follicular phase I. All groups were monitored daily and, in the presence of ovarian follicles smaller than the dominant size at the end of treatment, all were superstimulated with 1000 IU eCG. For plasma progesterone concentration recording, daily blood samples were collected from days −1 to 5 in the treated females in Group A and B. No significant differences were observed regarding the inhibition of follicle growth and in the plasma progesterone concentrations between Group A and B. The ovarian response to superstimulation was 56.2%, 71.4% and 90%, with the average number of dominant follicles produced per female being 4.4 ± 0.9; 4.8 ± 0.7 and 4.6 ± 0.6 in Groups A, B and C, respectively. The embryo recovery rate was 77.7%; 90% and 66.7% and the average number of embryos recovered per female was 2.9 ± 0.9; 2.6 ± 0.9 and 2.4 ± 0.8 for Groups A, B and C, respectively. In Groups A and B, the static follicular phase (III) seemed to be ideal for initiating the assisted reproductive technique of MOET. Although prior administration of P4 + EB seems to have no effect on the number of females that responded to the superstimulation treatments, the number of embryos recovered showed a tendency to be higher when ovarian follicle growth inhibition was performed beforehand.

1. Introduction

In the last few years the demand for South American camelids has increased worldwide due to the characteristics of their fibre and their use as pets (especially llamas). It has thus become of interest to develop biotechnologies that

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can be applied to upgrade the genetic make up of llamas. For this reason, it is essential to identify superior animals and use them in *in vivo* and *in vitro* embryo production and transfer programmes and thus accelerate the distribution of the genome in the population. These accelerated reproductive techniques could possibly also be employed as a model for application in wild species, such as the guanaco and vicuna for the preservation of their genetic material.

Camelids possess unique reproductive characteristics that constitute a challenge for the development of assisted reproductive techniques. Llamas are monotocous and the length of their gestation period varies between 342 and 350 days—thus the average number of progeny any female can produce throughout her reproductive life is limited to spread a desired genome effectively. The multiple ovulation and embryo transfer (MOET) technique allows an alternative to this limitation—to surpass and reduce the generation interval. Various gonadotrophin hormones have been used in such superstimulatory treatments, e.g. FSH and eCG. However the effectiveness of a single administration of eCG makes it the most convenient in the reproductive management procedure, when compared to FSH, which has a shorter half-life and therefore needs repeated doses over a period of time (Bravo et al., 1995; Agüero et al., 2001).

Greater efficiency has been recorded when superstimulatory treatments are initiated when the ovarian follicle development is minimal, and various protocols using progesterone have been developed over time in llamas to control the ovarian follicular dynamics. Alberio and Aller (1996) carried out a trial using 50 mg injectable progesterone over a period of 12 days. This dose induced a reduction in follicle diameter to below 5 mm on day 7 of treatment and plasma progesterone levels of 9.4 ng/ml on day 6. Chaves et al. (2002), treating with CIDR®s (devices 0.33 g progesterone) for a period of 16 days succeeded in decreasing the average follicle diameter from day 5 to reach a maximum plasma progesterone level (30 nmol/l) on day 1. Similarly Cavilla et al. (2006) used an intravaginal device (Cue-mate®; 0.78 g progesterone) for 6 days, inducing a reduction in ovarian follicle diameter during this period—with the absence of follicles of ovulatory size at the time of device removal. During this hormonal study, circulating progesterone concentrations were maximum on day 2 (5.88 ± 0.46 ng/ml) and then began to gradually decline—remaining above 2 ng/ml until day 6 of treatment and decreasing to basal values on the day after intravaginal device removal. Combinations of progesterone and estrogens have also been used to control ovarian follicle dynamics in camelids (Ratto et al., 2003; Trasorras et al., 2005).

The objective of this study was to evaluate the embryo recovery rate in superstimulated llamas which had been treated with injectable progesterone and estradiol benzoate to inhibit follicle growth prior to superstimulation.

2. Materials and methods

A total of 50 non-pregnant, non-lactating llamas, between 5 and 8 years of age and recording a mean body weight of 100 ± 25 kg were used in this study. All animals were in a good nutritional status (body condition) and both healthy and reproductively active at the time of the trial. The study was carried out at the Faculty of Veterinary Sciences of the University of Buenos Aires, Argentina; 35° South latitude. Females were kept separate from the males and fed bales of grass, concentrates ad lib and had free access to water.

2.1. Inhibition of ovarian follicular growth

Daily monitoring via rectal palpation and ultrasound (Berger® LC 2010 with a 5 MHz linear transducer), commenced 2 days prior to the treatment and all females were classified according to their ovarian follicular phase in the following classes:

- *Phase I*: growth phase, with follicles <7 mm in diameter ($n = 10$).
- *Phase II*: growing follicles >7 mm (dominant follicle) ($n = 10$).
- *Phase III*: follicles in a static phase (with variations in follicle diameter of only 1 mm in two consecutive measurements) ($n = 10$).
- *Phase IV*: follicles in regression (decrease in follicle diameter in subsequent measurements) ($n = 10$).

Females were randomly divided into Groups A and B for each phase, with five females being allocated to Group A and five to Group B in each follicular phase.

Females from Group *A* ($n = 20$) received a single dose of 1 mg of estradiol benzoate (EB) (Estradiol 10®, Laboratorio Allignani, Argentina) on the first day of the treatment + 100 mg of progesterone (P4) (Progesterona®, Laboratorio Allignani, Argentina) i.m. for 5 consecutive days prior to superstimulation. Females in Group *B* ($n = 20$) received a single dose of 1 mg of EB on the first day of the treatment + 150 mg P4, i.m. for 5 consecutive days prior to superstimulation.

Ovarian monitoring with the aid of ultrasonography was continued over the 5-day treatment period and the presence of follicles $\leq$5.5 mm on day 5 of treatment was considered to be a positive response (Fig. 1).

Group C ($n = 10$) did not receive any hormonal treatment prior to superstimulation and was monitored using ultrasound until the presence of follicles smaller than the dominant size were detected (phase I) and at this time superstimulatory treatment was initiated.

2.2. Plasma progesterone assay

For plasma progesterone concentration determinations, daily blood samples were collected from days −1 to 5 in the treated females of Groups A and B, using heparin-coated tubes and the blood collected via jugular veni-puncture. Blood samples were centrifuged immediately after collection to recover the plasma. Plasma was then stored at −18 °C until analyzed for plasma progesterone concentration. A RIA kit (DPC, Los Angeles, CA, USA), previously validated for llama plasma was used for the hormonal determinations (Bianchi et al., 2007).

2.3. Ovarian superstimulation

Superstimulatory treatment was started in females from Groups A and B that showed a positive response to the inhibition of follicle growth on the last day of treatment (day 5) and all females in Group C (control). A single dose of 1000 IU eCG (Novormon 5000®, Laboratorio Syntex, Bs. As. Argentina) was administered i.m. to all females to induce superovulation.

Ultrasound monitoring of the ovaries was carried out daily to determine the ovarian superstimulatory response. The presence of two or more pre-ovulatory follicles was considered a positive response, with pre-ovulatory follicles $\geq$7 mm.

2.4. Embryo recovery

Females that exhibited a positive response to the superstimulatory treatment were mated to two different llama males, 24 h apart. Together with the first mating, 8 μg Buserelin (Receptal®, Laboratorio Hoechst, Bs. As., Argentina) was administered intravenously (Fig. 1) to facilitate ovulation.

On day 8 after mating, the uterus of each female was flushed with D-PBS, supplemented with 1% inactivated Fetal Calf Serum (FCS), using a transcervical Foley-type catheter. The recovered media was filtered using a sterile EnCom filter and embryos washed three times in D-PBS, supplemented with 10% inactivated FCS. The embryos were microscopically evaluated according to the IETS classification of embryo morphology (International Embryo Transfer Society) (Robertson and Nelson, 1998).