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Transfer of cooled llama embryos obtained from synchronized females

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ABSTRACT

This study evaluated the efficiency of a synchronization protocol based on GnRH and $PGF_{2\alpha}$ on embryo donor llamas for fixed timed mating and assessed the viability of embryos maintained at 5 °C and 15 °C for 24 h, using the Equitainer® and the Botu-BOX® as cooling devices respectively. Llamas were divided into four follicular wave groups: growth, dominance, static and regression; they received a GnRH analogue on day 0 followed by a second dose plus cloprostenol on day 8 and 15 and mating was indicated in females with a follicle ≥ 6 mm. Embryos were recovered 8 days post mating. Synchronization rate was 80% for the treated embryo donors, with no significant differences among groups. Uterine flushing was performed in 70% of the treated females (87.5% of mated llamas) and an embryo was recovered in 50%. Fourteen embryos were assigned randomly to 5 °C (Equitainer® group) and 15 °C (Botu-BOX® group) preservation for 24 h to be transferred later. In the Equitainer® group, we obtained 14% pregnancies and a female offspring was born. In the Botu-BOX® group, 28% resulted pregnant but subsequently pregnancies were lost. This protocol was effective for synchronizing follicles in growth phase in 80% of embryo donor llamas. In addition, cooling llama embryos using the Equitainer® and the Botu-BOX® as cooling devices to 5 °C and 15 °C respectively, preserves its morphology and viability for 24 h.

1. Introduction

In recent years, interest in South American camelids (SAC) has increased, not only in the textile industry for the quality of their fibre and in the food industry for the quality of their meat, but also in the pharmaceutical and biotechnological industries due to the unique properties of their antibodies (Desmyter et al., 2015; Hamers-Casterman et al., 1993). Llama antibodies are the smallest molecules in nature with antigen-binding capacity, offering a number of advantages compared to conventional antibodies for therapeutic applications. They are currently being developed against SARS-CoV-2 to combat COVID-19 as either a preventive or curative therapeutic alternative (Ezzikouri et al., 2022; Huo et al., 2020; Wrapp et al., 2020; Xu et al., 2021; Yuan et al., 2017). Thus, research in reproduction in these species has also increased, primarily in llamas (*Lama glama*) and alpacas (*Vicugna pacos*).

An important peculiarity of SAC is that they are induced ovulators, requiring copulation (England et al., 1969; Fernandez-Baca et al., 1970) or the administration of human chorionic gonadotropin (hCG), GnRH or their analogues (buserelin or deslorelin) (Adam et al., 1992) to induce the ovulation of a dominant follicle. In non-mated females, ovarian activity occurs in waves of follicular growth and regression. The time required to complete a wave in llamas is between 22 and 24 days with a growing phase during the first 9–10 days (including the dominance phase), a plateau phase the next 5 days and a regression phase for the last 8–9 days (Cavilla et al., 2013; Chaves et al., 2002). Regression and growth of successive dominant follicles might overlap (Cavilla et al., 2013; Bravo et al., 1990), so that as one dominant follicle is regressing, another one is growing to dominance. As a result, unmated females may remain almost with constant receptivity (England et al., 1969). Nevertheless, not all follicular waves show overlapping (Bravo et al., 1990), resulting in periods of time when females are not receptive. Furthermore, there is no association between receptivity and presence of a

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dominant follicle or plasma oestradiol concentration (Fernandez-Baca et al., 1970; Bravo et al., 1990; Vaughan et al., 2003). Although most camelid producers indicate natural mating to females that show sexual receptivity (Vaughan et al., 2003), it is known that conception rates achieved are lower (50–75%) than when mating is indicated in the presence of a dominant growing follicle detected by ultrasonography (Tibary, 2015). Therefore, to improve pregnancy rates it is important to mate the female when there is a growing or early static dominant follicle which contain oocytes with a greater capacity for fertilization (Bianchi et al., 2018; Gallelli et al., 2019; Vaughan, 2011). Thus, the development of a follicular wave synchronization protocol that ensures the presence of a dominant follicle, would allow the application of fixed-time natural mating or artificial insemination (AI) and embryo transfer (ET) programs in large herds of females, improving management practices and optimizing reproductive efficiency in order to satisfy the increasing demand of meat and fibre production from SAC (Gallelli et al., 2019; Trasorras et al., 2009; Zampini et al., 2020).

Ovulation occurs in llamas 30 ± 0,5h post mating (Bourke et al., 1992) and the embryo reaches the uterus approximately 6–6.5 days after ovulation (Picha et al., 2013). So effectively embryo descent to the uterus is between 7 and 7.5 days post mating (dpm) (Adam et al., 1992; Picha et al., 2013), and for this reason embryo recovery is performed by flushing the uterus between 7 and 8 dpm (Sumar, 2008; Sumar, 2013; Vaughan et al., 2013; Trasorras et al., 2017). Embryo cryopreservation enables genetic preservation, increases the intervals of transportation and allows for embryo storage until the time of transfer. Nonetheless, the variability in embryo size in camelids makes techniques such as freezing and vitrification difficult and has led to limited results (Herrid et al., 2017). Due to this difficulty, embryo cooling becomes a useful short term storage alternative as it is easy to implement and suitable to be applied in the field. This technique has been widely used in equine species for several years and the pregnancy rates using embryos cooled for 24 h, has comparable results to those obtained with embryos transferred immediately after recovery (Carnevale et al., 1987; Carnevale et al., 2000; Carney et al., 1991; Cook et al., 2010). There are several devices for cooled-embryo transportation currently in the veterinary market, the Equitainer® (Hamilton Biovet, USA) being the one with the highest distribution, but with a higher cost. This device takes approximately 10 h to reach its final internal temperature of 5 °C. Another type of device, with a lower cost, is the Botu-BOX® (Botupharma Biotecnologia Animal, Brazil) that lowers temperature to 15 °C in approximately 6 h and maintains it for 24 h. The application of embryo cooling would allow an increase in the embryo recovery/transfer interval, would avoid the need for the recipient female being in the same location as the donor female and would permit transportation of the embryo to a laboratory where embryo preservation via freezing or vitrification could be undertaken.

Thus, the objectives of this study were: 1) to synchronize embryo donor llamas using two doses of GnRHa and two doses of cloprostenol for scheduling fixed-time mating and 2) to evaluate the viability of embryos maintained at 5 °C and 15 °C for 24 h using the Equitainer® and the Botu-BOX® as cooling devices respectively, by the establishment of pregnancies after transfer to recipient females.

2. Materials and methods

2.1. Animals

Non-pregnant, non-lactating female llamas ($n = 43$) ranging between 4 and 12 years of age and with an average body weight of 120 ± 22 kg were used in this study. All animals were in good body condition and were healthy and reproductively active at the time of the trial. Females were kept separate from the males and fed with hay and water *ad libitum*. The study was conducted between March and November at the Faculty of Veterinary Sciences of the University of Buenos Aires, situated 34° 36′ S and 58° 26′ W at sea level. The climate in Buenos Aires is temperate and humid, without a marked daily temperature range but with very different seasons, having hot summers and cold winters. The average annual temperature is 18 °C (maximum 28 °C; minimum 8 °C). Rainfall is more frequent during the summer season, with an annual average of 1100 mm.

This study was approved by the Committee for the Use and Care of Laboratory Animals (CICUAL, 2017/67) of the Faculty of Veterinary Sciences of the University of Buenos Aires.

2.2. In vivo embryo production from single ovulations

2.2.1. Treatment groups: Follicular wave synchronization

Ovarian dynamics were monitored by transrectal palpation and ultrasonography (Berger LC 2010 plus with a 5 MHz linear-array electronic transducer), starting one day prior (−1) to the treatment and all embryo donor females were classified according to their ovarian follicular phase in one of the following groups ($n = 5$ each group):

- Group I: growing follicles < 7 mm in diameter.
- Group II: growing dominant follicles ≥ 7 mm in diameter.
- Group III: dominant follicles in a static phase (with variations in follicle diameter of only ≤ 0.5–1 mm in two consecutive measurements).
- Group IV: follicles in regression (decrease in follicle diameter in subsequent measurements).

The complete experimental design is described in Fig. 1. On day 0 (zero) each donor female received a single IV injection of 8 μg of a GnRHa (buserelin acetate; Receptal®, Intervet, Buenos Aires, Argentina) to induce endogenous LH release and ovulation. Eight days later, a second dose of GnRHa was injected, followed by a single IM injection of 250 μg of cloprostenol (Ciclase DL®, Syntex S.A., Buenos Aires, Argentina), with the objective of lysing the corpus luteum (CL) produced by the ovulation response to the first dose of GnRHa. On day 15, all groups received another IM injection of cloprostenol (a.m.) and only females with a follicle ≥ 6 mm (detected by ultrasound), received natural mating (p.m.).

Ovulation was controlled by an ultrasound examination two days after each GnRHa injection and was based on the disappearance of the follicle and observation of a CL on days 8, 15 and 23.

2.2.2. Control group

Ovarian dynamics were monitored by transrectal palpation and ultrasonography in the embryo donor females ($n = 9$) which were used as a control (no follicular wave synchronization). When a follicle in growth phase was detected (≥6 mm in diameter), natural mating was carried out (day 0). Ovulation was confirmed by ultrasound based on the disappearance of the dominant follicle 2 days post mating (dpm) and observation of a CL on day 8.

2.2.3. Natural mating

All donor females (treatment and control groups) were mated with a male of proven fertility. In all cases, a single mating was performed, with a mean duration of 25 min (range: 20 to 30 min). A total of 6 males were used (four matings per male; allowing at least a week of rest between each mating for any given male). In addition, ovulation was ensured in all donor llamas with a single IV injection of GnRHa immediately after mating.

2.3. Embryo recovery

Uterine flushing was carried out non-surgically 8 dpm, using a recto-vaginal approach in all mated donor llamas (Trasorras et al., 2010). Restless females were sedated with 0.2 mg/kg xylazine IV (Xilazina 10% ®, PRO-SER S.A., Buenos Aires, Argentina) before flushing. The manoeuvres were performed with the female either standing or in sternal

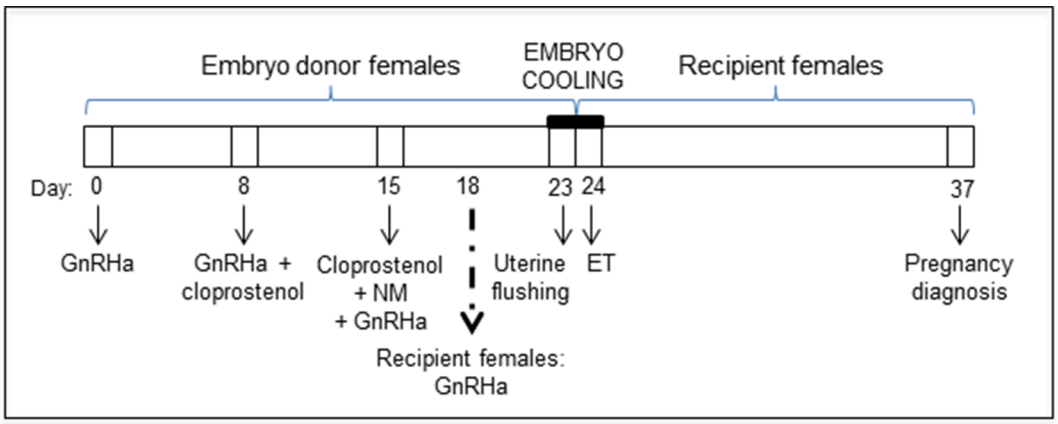

Fig. 1. Experimental design. NM: natural mating.

recumbency. The animal was restrained in stocks, the tail was wrapped and the rectum was emptied of faeces. The perineum was then scrubbed using a hypoallergenic detergent, rinsed carefully with clean water and then dried. A Foley catheter (12 or 16 Fr, according to female size) containing a stylet was inserted transcervically into the uterus and the catheter cuff was inflated cranial to the internal *cervical os* with 5 or 10 ml of air (according to catheter gauge) to keep it in place during recto-vaginal manipulation. The entire uterus was flushed 4 to 5 times with 30–35 °C Ringer's lactate solution, using a total volume of 500 ml. The recovered medium was filtered through a 70 μm embryo cup filter. After the flushing was finished, donor females received a single IM injection of cloprostenol to induce luteolysis.

2.4. Embryo management

2.4.1. Embryo evaluation

The residual medium in the embryo cup filter was transferred to a sterile plastic Petri dish and the embryos were searched for using a stereomicroscope under laminar flow. Once the embryos were located, they were transferred into a 4-well dish (IMV, L'Aigle, France) and washed by 2 or 3 successive passages in Syngro® Holding embryo maintenance medium (Vetoquinol, USA) and morphologically graded using the scale from 1 to 5 proposed by (Tibary and Anouassi, 1997). Only morphologically normal (grades 1 and 2) hatched blastocysts were included in the study. Once classified, embryo diameters were measured (μm).

2.4.2. Embryo cooling

Each embryo was placed inside a cryovial with Syngro® Holding medium. Cryovials, containing one embryo per vial, were placed into a 50 ml tube with warmed collection medium, so that the temperature drop was gradual (Moussa et al., 2006). The tubes were placed inside an Equitainer® (5 °C, Equitainer® group) or inside a Botu-BOX® (15 °C, Botu-BOX® group) for 24 h.

2.5. Transfer of in vivo produced embryos

2.5.1. Management of the recipient female

Synchronization of recipient females using a single injection of GnRHa in the presence of a dominant follicle was conducted 3 dpm the donor female. Ovulation was confirmed using transrectal ultrasonography indicated by the disappearance of the dominant follicle two days later and observation of a CL on day 6.

2.5.2. Transcervical embryo transfer technique

Transcervical embryo transfer (ET) was performed in recipient females on day 6 after GnRHa administration (Trasorras et al., 2010). The preparation of recipient females was the same as for donor females. A lubricated gloved hand was inserted in the rectum to hold the cervix while an assistant separated the vulva labia and an ET pipette, covered with a sterile sheath (IMV® ET Sheath, 21″, France) and carrying the 0.25 ml straw (IMV® ET Straws, France) containing a single embryo, was inserted into the vagina. Cervical threading was performed aided by transrectal manipulation and the embryo was deposited in the uterine horn ipsilateral to the CL.

2.5.3. Pregnancy diagnosis

Pregnancy diagnosis was performed 13 days after ET, by transrectal ultrasonographic visualization of the 22 days old embryonic vesicle and the embryo proper. Viability was confirmed by the presence of a heartbeat 3 days later (day 25 of pregnancy) (Sumar, 2013; Bravo et al., 2000).

2.6. Statistical analysis

The percentage of donor females that ovulated in response to the first and second injection of GnRHa was compared using a Pearson Chi-square test. The mean follicular diameter for each group of females at day 15 of the protocol was evaluated using a nonparametric Kruskall Wallis test. The number of pregnancies obtained was compared between the Equitainer® group and Botu-BOX® group using Fisher's exact test and $P < 0.05$ were considered significant.

3. Results

3.1. Ovarian follicular synchronization

Table 1 shows the mean follicular diameter on day 0 and day 8 in each group of females and the percentages of ovulation in response to GnRHa for each day, respectively. After the first injection of GnRHa on

Table 1
Ovulatory response according to the stage of follicular development in llamas injected with GnRHa on days 0 and 8 (mean ± SD).

Group	Mean follicle diameter on day 0 (mm)	Percentage of females that ovulated after day 0 of GnRHa treatment (N° ovulated females/total)	Mean follicle diameter on day 8 (mm)	Percentage of females that ovulated after day 8 of GnRHa treatment (N° ovulated females/total)
I	3.6 ± 2.5	60 (3/5)	6.4 ± 1.8	80 (4/5)
II	8.3 ± 0.6	80 (4/5)	7.6 ± 1.6	40 (2/5)
III	8.7 ± 2.1	80 (4/5)	4.8 ± 2.2	40 (2/5)
IV	7.5 ± 1.6	60 (3/5)	5.9 ± 3.2	80 (4/5)

day 0, groups I and IV tended to have lower ovulation percentages (60% in each) compared to groups II and III (80% in each), even though no significant differences were detected ($P > 0.05$). Conversely on day 8, after the second injection of GnRHa, the percentages of ovulation tended to be higher in groups I and IV (80% in each) compared to groups II and III (40% in each), although again not significantly different ($P > 0.05$).

The mean follicular diameter in each group by day 15 of the protocol, the percentage of females that received natural mating, the percentage of females with CL on the day of uterine flushing and the percentage of females with a positive embryo recovery can be observed in Table 2. After the administration of two doses of GnRHa, separated by 8 days, and two doses of cloprostenol, separated by 7 days, 80% of the females (16/20) presented an ovulatory follicle by day 15 (mean follicular diameter of 6.2 ± 1.7 mm), without significant differences between groups ($P > 0.05$). Synchronized females (80%) received natural mating and GnRHa.

3.2. Embryo retrieval rates

Uterine flushing was performed in 70% (14/20) of the treated females. Of the synchronized females that received natural mating, 87.5% (14/16) presented a CL in one of their ovaries by day 23, therefore transcervical flushing was performed and an embryo was recovered in 50% (7/14) of the llamas. The two remaining mated females developed cystic follicles instead of a CL, so they were not flushed.

The embryo recovery rate in the control group was 78% (7/9).

The fourteen recovered embryos were at the hatched blastocyst stage and their size ranged from 800 to 1800 μm, all of them of embryo quality grade 1 and 2.

3.3. Embryo cooling

The embryos were randomly and equally assigned for cooling, to either the Equitainer® group (5 °C; $n = 7$; 800 to 1800 μm) or the Botu-BOX® group (15 °C; $n = 7$; 800 to 1500 μm). The embryo cooling method can be observed in Fig. 2. After storage for 24 h, the quality and the diameter of the embryos from both groups were maintained but just one embryo from Equitainer® group of 1800 μm had reduced its diameter to 1500 μm but remained of grade 1 quality.

3.4. Embryo transfer and pregnancy diagnosis

After 24 h, each embryo was transferred to one recipient female ($n = 14$).

In the Equitainer® group, one female was confirmed pregnant (1/7; 14%). The grade 1 hatched blastocyst 1300 μm had been transferred to the right uterine horn, ipsilateral to the CL. This pregnancy was maintained during 11 months and a female offspring was born. In the Botu-BOX® group, two females were diagnosed pregnant (2/7; 28%). Both embryos had been transferred to the left uterine horn, ipsilateral to the CL, one of them (grade 1 hatched blastocyst; 900 μm) resulting in embryonic loss one month after pregnancy diagnosis and the other (grade 2 hatched blastocyst; 1300 μm), in a pregnancy loss after two months. There were no significant differences in pregnancy rates between cooling methods ($P > 0.05$).

Table 2
Results obtained after ovarian synchronization treatment in embryo donor llamas that received GnRHa on days 0 and 8, cloprostenol on days 8 and 15 and natural mating on day 15. Data are mean ± SD.

Group	Mean follicle diameter on day 15 (mm)	Percentage of mated females (N° mated females/total)	Percentage of females with a CL on the day of uterine flushing (N° females with CL/N° mated females)	Percentage of females with a positive embryo recovery (N° embryos recovered/N° flushed females)
I	7.3 ± 1.9	80 (4/5)	100 (4/4)	50 (2/4)
II	5.9 ± 0.6	60 (3/5)	100 (3/3)	66.7 (2/3)
III	7.1 ± 2.9	80 (4/5)	50 (2/4)	50 (1/2)
IV	6.4 ± 0.3	100 (5/5)	100 (5/5)	40 (2/5)
Total (n = 20)	6.2 ± 1.7	80 (16/20)	87.5 (14/16)	50 (7/14)

4. Discussion

The results obtained in the present study demonstrated that application of two doses of GnRHa separated by 8 days, together with two doses of cloprostenol separated by 7 days, synchronizes the emergence of a new follicular wave by day 15 and the appearance of a follicle in growth or dominance phases in 80% of the llamas, independently of the follicular phase of each female at the beginning of the treatment. Protocols have also been developed based on the administration of two doses of GnRHa separated by 14 days in dromedaries (Skidmore et al., 2009) or separated by 10 days in llamas (Bianchi et al., 2018), with one dose of cloprostenol on day 7 in both species. In these two studies, the average emergence of a new follicular wave after the first administration of GnRHa was on day 4.5 for dromedaries and on day 3 for llamas, though certain variability was observed, depending on the phase of the follicular wave of each animal. A 73% synchronization rate was achieved in dromedaries and 66% synchronization rate in llamas; that is, they presented a dominant follicle at the time of the second GnRHa injection.

According to (Bravo et al., 1991), the ovulation induced by natural mating would occur only in females with follicles of 7 to 12 mm in diameter, while in those with smaller follicles, in the growth phase, a lower increase of LH was observed, without altering normal follicular development. Recently, (Bianchi et al., 2018) informed that growing follicles of 5.5 mm in diameter responded to GnRHa injection (without mating), increasing their size to 6.5 mm at 24 h and ovulating 48 h post administration. Our results showed, similar to the observation of Bianchi et al., that females with an average follicle diameter of 6.2 mm and that received natural mating plus GnRHa injection on day 15, responded with ovulation and the formation of a CL (87.5%; 14/16). Probably, the combination of natural mating together with the GnRHa injection, accelerated the growth of 6 mm follicles and enabled the events necessary for ovulation to occur. Recovering an embryo in 50% (7/14) of these females after transcervical uterine flushing, would indicate that after natural mating plus GnRHa, the follicles can contain a mature oocyte with fertilizing capacity. Thus, this treatment would permit scheduled fixed-time matings, it could be used in AI programs or even to synchronize donor females with recipients in ET programs, as it is a simple protocol that would allow one to dispense with the use of ultrasonography in the field.

Of the 40 induced ovulations registered during this study, 22 occurred in the left ovary (55%) and 18 in the right ovary (45%), thus showing a similar occurrence of dominant follicles in both ovaries as that observed by (Chaves et al., 2002) (53% in the left ovary and 47% in the right ovary). We also observed the appearance of two double ovulations (2/40; 5%), which has been reported to commonly occur in animals with good health and body condition (Campbell et al., 2015).

In llamas and alpacas, achieving percentages of 40–50% pregnancy after the transfer of fresh embryos is considered a satisfactory result (Vaughan et al., 2013; Trasorras et al., 2010; Del Campo et al., 1995; Taylor et al., 2000). Until now, few studies have been conducted in cooling camelid embryos. In this work we observed that cooling llama blastocysts to 5 °C (Equitainer® group) and 15 °C (Botu-BOX® group), maintained the morphological characteristics (embryo quality and size) for 24 h. This is consistent with studies conducted in dromedaries where,

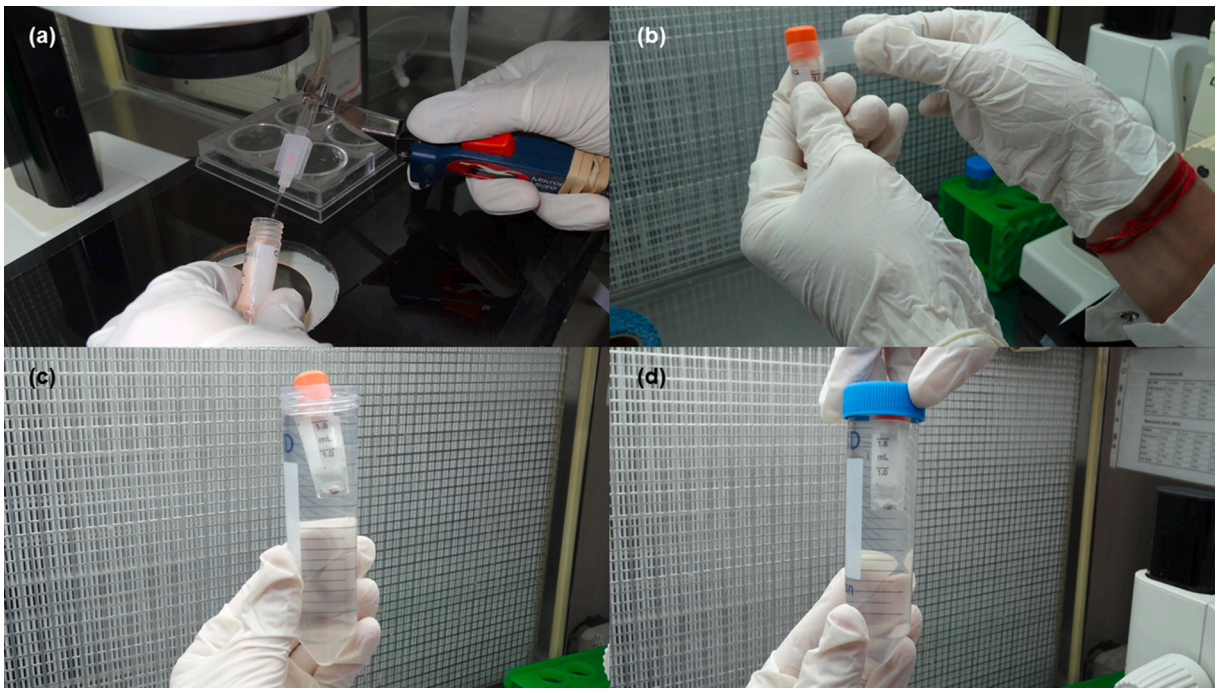

Fig. 2. Embryo cooling method. (a) Each embryo was placed inside a cryovial with Syngro® Holding medium. (b) The cryovial was sealed with PARAFILM®. (c) The sealed cryovial was placed in a 50 ml tube containing warmed collection medium. (d) The tube was closed with the cryovial within, making sure there was no air inside. Then the tube, containing the cryovial with the embryo inside, was placed into the corresponding cooling device.

after cooling embryos to 4 °C for between 24 h (Skidmore et al., 2002; Abd-Elfattah et al., 2020) and 120 h (Abd-Elfattah et al., 2020), no evident morphological changes were found. According to our results, cooling also maintained embryo viability for 24 h, achieving a 14% and 28% pregnancy rate in the llamas that received blastocysts stored at 5 °C and 15 °C, respectively. In 2002, (von Baer et al., 2002) reported the transfer of 3 llama blastocysts cooled to 4 °C during 12 h (unspecified device), achieving a pregnancy. Likewise, dromedary blastocysts were cooled to 4 °C for 24 h using different devices, achieving 63% (20/32) pregnancy and calving (Equitainer®) (Skidmore et al., 2002) and 37% (10/27) pregnancy rate at 60 days post-transfer (refrigerator) (Abd-Elfattah et al., 2020). In turn, (Horteloup, 2012) maintained llama embryos during 18 h at 5 °C (n = 20) in an Equitainer® and at 25 °C (n = 22) in a different device designed for equine semen transportation (EquiPro®; Minitube, Germany), resulting in a 40% and a 63.6% pregnancy rate after 21 days, respectively; however they did not inform the birth of offspring. (Aller et al., 2015) reported a 21.5% pregnancy rate (3/14) after transfer of embryos cooled to 5 °C for 24 h and achieved the birth of 3 llama offspring. Similarly, we achieved the birth of a live offspring from one of the llamas in the Equitainer® device group (5 °C). Nevertheless, the 2015 study does not give details of the cooling device used. In the present study, embryo cooling to 15 °C using the Botu-BOX® device, which is inexpensive and accessible, proved to also maintain the viability of embryos during 24 h achieving a 28% (2/7) pregnancy rate after embryo transfer. It would be interesting to increase the number of embryo transfers using embryos maintained at 15 °C, with the objective of achieving live offspring with this simple conservation method.

5. Conclusion

The administration of two doses of GnRHa separated by 8 days together with two doses of cloprostenol separated by 7 days, synchronizes the emergence of a new follicular wave in llamas. Of the treated animals 80% presented a new follicle, in growth or dominant phase, 15 days after beginning the treatment, regardless of the phase of the follicular wave on day 0. The combined effect of natural mating plus GnRHa in females with an average 6.2 mm growing follicles, leads not only to ovulation and CL formation, but also fertilization and embryo recovery.

Regarding llama embryo preservation, using the Equitainer® and the Botu-BOX® as cooling devices to 5 °C and 15 °C respectively, allows morphology and viability to be maintained for 24 h. According to the results obtained so far, we achieved the first llama pregnancy after transfer of embryos stored in Botu-BOX® at 15 °C for 24 h, and llama offspring could be obtained from the transfer of embryos cooled to 5 °C in Equitainer® for 24 h.

Ethical statement

This study was approved by the Committee for the Use and Care of Laboratory Animals (CICUAL, 2017/67) of the Faculty of Veterinary Sciences of the University of Buenos Aires.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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References

Abd-Elfattah, A., Agag, M., Nasef, M., Muthukumaran, S., El-Raey, M., El-Khawaga, A., et al., 2020. Preservation of dromedary camel embryos at 4 °C for up to 5 days: Factors affecting the pregnancy and pregnancy loss rates. Theriogenology 143, 44–49. https://doi.org/10.1016/j.theriogenology.2019.11.024.

Adam, C.L., Bourke, D.A., Kyle, C.E., Young, P., McEvoy, T., 1992. Ovulation and embryo recovery in the llama. In: Proceedings of the 1st International Camel Conference, pp. 125–127.

Aller, J.F.F., Abalos, M.C.C., Acuña, F., Virgili, R., Requena, F., Cancino, A.K.K., 2015. Birth of live llama (*Lama glama*) derived from embryo transfer storage at 5°C for 24h. Small Rumin. Res. 132, 99–102. https://doi.org/10.1016/j.smallrumres.2015.10.007.

Bianchi, C.P., Benavente, M.A., Simonetti, M., Aba, M.A., 2018. Synchronization of time of ovarian follicular development in llamas (*Lama glama*) using a protocol based on GnRH and $PGF_{2\alpha}$. Anim. Reprod. Sci. 192, 200–205. https://doi.org/10.1016/j.anireprosci.2018.03.011.

Bourke, D.A., Adam, C.L., Kyle, C.E., McEvoy, T.G., Young, P., 1992. Ovulation, superovulation and embryo recovery in llamas. Proceedings of the 12th International Congress of Animal Reproduction 1:193–5.

Bravo, P.W., Fowler, M.E., Stabenfeldt, G.H., Lasley, B.L., 1990. Ovarian follicular dynamics in the llama. Biol. Reprod. 43, 579–585. https://doi.org/10.1095/BIOLREPROD43.4.579.

Bravo, W.P., Mayta, M.M., Ordoñez, C.A., 2000. Growth of the conceptus in alpacas. Am. J. Vet. Res. 61, 1508–1511. https://doi.org/10.2460/ajvr.2000.61.1508.

Bravo, P.W., Stabenfeldt, G.H., Lasley, B.L., Fowler, M.E., 1991. The effect of ovarian follicle size on pituitary and ovarian responses to copulation in domesticated South American camelids. Biol. Reprod. 45, 553–559. https://doi.org/10.1095/biolreprod45.4.553.

Campbell, A.J., Pearson, L.K., Spencer, T.E., Tibary, A., 2015. Double ovulation and occurrence of twinning in alpacas (*Vicugna pacos*). Theriogenology 84, 421–424. https://doi.org/10.1016/j.theriogenology.2015.03.027.

Carnevale, E.M., Squires, E.L., McKinnon, A.O., 1987. Comparison of Ham's F10 with CO^2 or Hepes buffer for storage of equine embryos at 5 C for 24 H. J. Anim. Sci. 65, 1775–1781. https://doi.org/10.2527/JAS1987.6561775X.

Carnevale, E.M., Ramirez, R.J., Squires, E.L., Alvarenga, M.A., Vanderwall, D.K., McCue, P.M., 2000. Factors affecting pregnancy rates and early embryonic death after equine embryo transfer. Theriogenology 54 (6), 965–979.

Carney, N.J., Squires, E.L., Cook, V.M., Seidel, G.E., Jasko, D.J., 1991. Comparison of pregnancy rates from transfer of fresh versus cooled, transported equine embryos. Theriogenology 36 (1), 23–32.

Cavilla, M.V., Bianchi, C.P., Maistruarena, C., Aba, M.A., 2013. Ultrasonographic and endocrine characterization of follicular waves in llamas with a special reference to the overlapping phenomenon during successive waves. Reprod. Domest. Anim. 48, 923–930. https://doi.org/10.1111/rda.12187.

Chaves, M.G., Aba, M., Agüero, A., Egey, J., Berestin, V., Rutter, B., 2002. Ovarian follicular wave pattern and the effect of exogenous progesterone on follicular activity in non-mated llamas. Anim. Reprod. Sci. 69 (1-2), 37–46.

Cook, V.M., Squires, E.L., McKinnon, A.O., Bailey, J., Long, P.L., 2010. Pregnancy rates of cooled, transported equine embryos. Equine Vet. J. 21, 80–81. https://doi.org/10.1111/J.2042-3306.1989.TB04684.X.

Del Campo, M.R., Del Campo, C.H., Adams, G.P., Mapletoft, R.J., 1995. The application of new reproductive technologies to South American camelids. Theriogenology 43, 21–30. https://doi.org/10.1016/0093-691X(94)00007-H.

Desmyter, A., Spinelli, S., Roussel, A., Cambillau, C., 2015. Camelid nanobodies: Killing two birds with one stone. Curr. Opin. Struct. Biol. 32, 1–8. https://doi.org/10.1016/j.sbi.2015.01.001.

England, B.G., Foote, W.C., Matthews, D.H., Cardozo, A.G., Riera, S., 1969. Ovulation and corpus luteum function in the llama (*Lama glama*). J. Endocrinol. 45, 505–513. https://doi.org/10.1677/JOE.0.0450505.

Ezzikouri, S., Nourlil, J., Tsukiyama-Kohara, K., Kohara, M., El Ossmani, H., Windisch, M.P., Benjelloun, S., 2022. Nanobodies: an unexplored opportunity to combat COVID-19. J. Biomol. Struct. Dyn. 40 (7), 3129–3131. https://doi.org/10.1080/07391102.2020.1845801.

Fernandez-Baca, S., Madden, D.H.L., Novoa, C., 1970. Effect of different mating stimuli on induction of ovulation in the alpaca. J. Reprod. Fertility 22, 261–267. https://doi.org/10.1530/jrf.0.0220261.

Gallelli, M.F., Bianchi, C., Trasorras, V., Zampini, E., Aba, M., Miragaya, M., 2019. Synchronization of time of development of ovarian follicular waves in South American Camelids. Anim. Reprod. Sci. 208, 106105. https://doi.org/10.1016/j.anireprosci.2019.06.017.

Gallelli, M.F., Bianchi, C., Trasorras, V., Zampini, E., Aba, M., Miragaya, M., 2019. Strategies for follicular wave synchronization in llamas. Taurus 21.

Hamers-Casterman, C., Atarhouch, T., Muyldermans, S., Robinson, G., Hammers, C., Songa, E.B., Bendahman, N., Hammers, R., 1993. Naturally occurring antibodies devoid of light chains. Nature 363 (6428), 446–448. https://doi.org/10.1038/363446a0.

Herrid, M., Vajta, G., Skidmore, J.A., 2017. Current status and future direction of cryopreservation of camelid embryos. Theriogenology 89, 20–25. https://doi.org/10.1016/j.theriogenology.2016.10.005.

Horteloup, M.P., 2012. Effect of holding temperature on pregnancy rate of llama embryos. International Congress of Animal Reproduction (ICAR), Satellite Meeting on Camelid Reproduction, p. 100-103.

Huo, J., Le Bas, A., Ruza, R.R., Duyvesteyn, H.M.E., Mikolajek, H., Malinauskas, T., Tan, T.K., Rijal, P., Dumoux, M., Ward, P.N., Ren, J., Zhou, D., Harrison, P.J., Weckener, M., Clare, D.K., Vogirala, V.K., Radecke, J., Moynié, L., Zhao, Y., Gilbert-Jaramillo, J., Knight, M.L., Tree, J.A., Buttigieg, K.R., Coombes, N., Elmore, M.J., Carroll, M.W., Carrique, L., Shah, P.N.M., James, W., Townsend, A.R., Stuart, D.I., Owens, R.J., Naismith, J.H., 2020. Neutralizing nanobodies bind SARS-CoV-2 spike RBD and block interaction with ACE2. Nat. Struct. Mol. Biol. 27 (9), 846–854. https://doi.org/10.1038/s41594-020-0469-6.

Moussa, M., Duchamp, G., Daels, P.F., Bruyas, J.-F., 2006. Effect of embryo age on the viability of equine embryos after cooled storage using two transport systems. J. Equine Vet. 26 (11), 529–534.

Picha, Y., Tibary, A., Memon, M., Kasimanickam, R., Sumar, J., 2013. Chronology of early embryonic development and embryo uterine migration in alpacas. Theriogenology 79, 702–708. https://doi.org/10.1016/j.theriogenology.2012.11.027.

Skidmore, J.A., Billah, M., Allen, W.R., 2002. Investigation of factors affecting pregnancy rate after embryo transfer in the dromedary camel. Reprod. Fertil. Dev. 14, 109–116. https://doi.org/10.1071/rd01100.

Skidmore, J.A., Adams, G.P., Billah, M., 2009. Synchronisation of ovarian follicular waves in the dromedary camel (Camelus dromedarius). Anim. Reprod. Sci. 114, 249–255. https://doi.org/10.1016/j.anireprosci.2008.08.024.

Sumar, J.B., 2008. Alpacas finas obtenidas en vientres de llama por transferencia de embriones. Agronoticias 132–134.

Sumar, J.B., 2013. Embryo transfer in domestic South American camelids. Anim. Reprod. Sci. 136, 170–177. https://doi.org/10.1016/j.anireprosci.2012.10.029.

Taylor, S., Taylor, P.J., James, A.N., Godke, R.A., 2000. Successful commercial embryo transfer in the llama (*Lama glama*). Theriogenology 53, 344. https://doi.org/10.1016/S0093-691X(01)00676-8.

Tibary, A., 2015. Transferencia de embriones en camélidosEMBRYO TRANSFER IN CAMELIDS. Transferencia de embriones en camélidos. SPERMOVA 5 (2), 234–252. https://doi.org/10.18548/aspe/0002.44.

Tibary, A., Anouassi, A., 1997. Theriogenology in Camelidae. Anatomy, physiology, pathology and artificial breeding. In: Reproductive Physiology in female camelidae. United Arab Emirates. Rabat, Marocco: Actes Éditions, Institut Agronomique et Vetérinaire Hassan II (Maroc), printed by Abu Dhabi Printing and Publishing Company.

Trasorras, V.L., Chaves, M.G., Miragaya, M.H., Pinto, M., Rutter, B., Flores, M., et al., 2009. Effect of eCG superstimulation and buserelin on cumulus-oocyte complexes recovery and maturation in Llamas (*Lama glama*). Reprod. Domest. Anim. 44, 359–364. https://doi.org/10.1111/j.1439-0531.2007.00972.x.

Trasorras, V.L., Chaves, M.G., Neild, D.M., Gambarotta, M., Aba, M.A., Agüero, A., 2010. Embryo transfer technique: Factors affecting the viability of the corpus luteum in llamas. Anim. Reprod. Sci. 121, 279–285. https://doi.org/10.1016/j.anireprosci.2010.06.004.

Trasorras, V.L., Carretero, M.I., Neild, D.M., Chaves, M.G., Giuliano, S.M., Miragaya, M. H., 2017. Production, preservation, and transfer of South American Camelid embryos. Front. Vet. Sci. 4 https://doi.org/10.3389/fvets.2017.00190.

Vaughan, J., 2011. Ovarian function in South American camelids (alpacas, llamas, vicunas, guanacos). Anim. Reprod. Sci. 124, 237–243. https://doi.org/10.1016/j.anireprosci.2010.08.031.

Vaughan, J.L., Macmillan, K.L., Anderson, G.A., D'occhio, M.J., 2003. Effects of mating behaviour and the ovarian follicular state of female alpacas on conception. Australian Vet. J. 81 (1-2), 86–90.

Vaughan, J., Mihm, M., Wittek, T., 2013. Factors influencing embryo transfer success in alpacas-A retrospective study. Anim. Reprod. Sci. 136, 194–204. https://doi.org/10.1016/j.anireprosci.2012.10.010.

von Baer, A., Del Campo, M.R., Donoso, D., Toro, F., von Baer, L., Montecinos, S., Rodriguez-Martinez, H., Palasz, A.T., 2002. Vitrification and cold storage of llama (*Lama glama*) hatched blastocysts. Theriogenology 57, 89.

Wrapp, D., De Vlieger, D., Corbett, K.S., Torres, G.M., Wang, N., Van Breedam, W., Roose, K., van Schie, L., Hoffmann, M., Pöhlmann, S., Graham, B.S., Callewaert, N., Schepens, B., Saelens, X., McLellan, J.S., 2020. Structural basis for potent neutralization of betacoronaviruses by single-domain camelid antibodies. Cell 181 (5). https://doi.org/10.1016/j.cell.2020.04.031.

Xu, J., Xu, K., Jung, S., Conte, A., Lieberman, J., Muecksch, F., Lorenzi, J.C.C., Park, S., Schmidt, F., Wang, Z., Huang, Y., Luo, Y., Nair, M.S., Wang, P., Schulz, J.E., Tessarollo, L., Bylund, T., Chuang, G.-Y., Olia, A.S., Stephens, T., Teng, I.-T., Tsybovsky, Y., Zhou, T., Munster, V., Ho, D.D., Hatziioannou, T., Bieniasz, P.D., Nussenzweig, M.C., Kwong, P.D., Casellas, R., 2021. Nanobodies from camelid mice and llamas neutralize SARS-CoV-2 variants. Nature 595 (7866), 278–282. https://doi.org/10.1038/s41586-021-03676-z.

Yuan, Y., Cao, D., Zhang, Y., Ma, J., Qi, J., Wang, Q., Lu, G., Wu, Y., Yan, J., Shi, Y.i., Zhang, X., Gao, G.F., 2017. Cryo-EM structures of MERS-CoV and SARS-CoV spike glycoproteins reveal the dynamic receptor binding domains. Nat. Commun. 8 (1) https://doi.org/10.1038/ncomms15092.

Zampini, E.G., Veiga, M.F., Fumuso, F.G., Cabido, L., Neild, D.M., Chaves, M.G., et al., 2020. Development of a GnRH-$PGF_{2\alpha}$ based synchronization and superstimulation protocol for fixed-time mating in llama embryo donors. Front. Vet. Sci. 7, 1–6. https://doi.org/10.3389/fvets.2020.595889.