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Short communication

In vitro embryo production in sheep: Pregnancy after long periods of oocyte and embryo transport

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ABSTRACT

To evaluate the feasibility of long distance transport of ovine oocytes and in vitro produced embryos. Santa Ines pluriparous sheep ($n = 5$) were synchronized with intravaginal devices containing 0.3 mg progesterone (CIDR®, Pfizer, Netherlands) for 10 days. Thirty-six hours prior to progesterone device removal and follicular aspiration, 0.04 mg D-cloprostenol sodium (Ciosin®, Coopers, Brasil), 200 IU FSH (Folltropin®, Bioniche, Canada) and 300 IU eCG (Novormon®, Syntex, Argentina) were administered intramuscularly. Oocyte retrieval was performed via mid-ventral laparotomy, under general anesthesia. The oocytes were evaluated and classified under a stereomicroscope and placed in cryovials with TCM 199, aerated with 5% CO_2, covered with mineral oil, sealed, wrapped with parafilm and stored in a transport incubator at 38.5 °C. Oocytes were then transported via the road for 14 h to the laboratory, where the oocytes then completed more than 10 h of IVM. In vitro fertilization (IVF) was performed with frozen sperm from a single ram. On Day 3 the embryos were placed in cryovials containing SOFovine (In Vitro Brasil, Sao Paulo, Brazil), aerated with 5% CO_2, covered with mineral oil, sealed, wrapped with parafilm and placed into the same portable incubator. The embryos were returned to the farm under the same transport conditions (km and h). Upon arrival at the farm, all embryos were evaluated and transferred into recipient ewes 4 days after the initial oocyte retrieval. Embryos were transferred in sets of 3 to 4, into the apex of the uterine horn, ipsilateral to the corpus luteum, by means of mid-ventral laparotomy, under general anesthesia. Pregnancy was diagnosed using transrectal ultrasonography 30 days after embryo transfer. On average 9.8 ± 5.7 oocytes (49 oocytes/5 donors) were obtained per donor, and 25 grade I morulae were transferred into 7 recipients. One pregnancy (14.3%) was confirmed resulting in a healthy lamb. These results indicate the feasibility of in vitro embryo production in sheep after maturation and embryonic development following transport over long distances.

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1. Introduction

The sheep industry is a growing enterprise in Brazil. The development of new animal reproduction biotechnologies will then allow acceleration of genetic improvement in the ovine breeds of interest in Brazil. When properly used, biotechnologies can contribute to achieving

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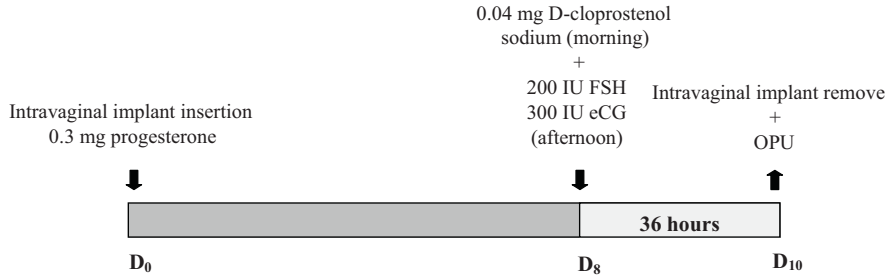

Fig. 1. One shot protocol administered pre-follicular aspiration in sheep.

significant improvements in productivity and profitability of flocks (Simplício et al., 2007).

Ovum pick up (OPU) is a reproductive biotechnology widely employed in several species, but its use in small ruminants is still very limited. Anatomical constraints represent an important limitation, due to the difficulty of performing ovarian manipulation through the vagina (Baldassarre et al., 1994, 1996; Graff et al., 1999). Another restriction is related to the use of pre-aspiration protocols that promote more effective control of follicular waves, and consequently lead to an increased number of oocytes being recovered. Both the complexity of the follicular dynamics in sheep and the anatomical characteristics that hinder access to the ovaries make the monitoring of ovarian sheep follicles difficult (Guinther et al., 1995; Bartlewski et al., 1999).

Despite numerous improvements in techniques that have been employed, many aspects still need to be improved, including the hormonal protocols for follicular aspiration, oocyte collection techniques and in vitro procedures (maturation, fertilization and culture until the time of embryo transfer). A common problem is often the large distances between the IVF laboratories and sheep farms. Additionally, there are no really efficient protocols for the cryopreservation of in vitro produced sheep embryos. Therefore, to overcome geographic distances other alternatives should be used to allow the transfer of fresh embryos. In bovine, there is already a strategy available for highly efficient transport of fresh oocytes and embryos over long distances (Pontes et al., 2010). Conversely, however, there are no similar reports for sheep,

In this context, the objective of this preliminary study was to evaluate the feasibility of oocyte maturation and embryonic development in a portable incubator following the transport of sheep oocytes and embryos over a long distance.

2. Materials and methods

The experiment was conducted in Londrina, the northern Paraná State of Brazil (23°30′26″S latitude, 51°14′06″W), at the onset of the natural breeding season. The climate is generally subtropical with peak rainfall in summer. During the experimental period, the animals were maintained on a pasture composed of star grass (*Cynodon pletostachyrus* Pilger) and thick bush grass (*Paspalum notatum*), with continuous grazing and access to minerals and water ad libitum. At night, the animals were brought into the fold, and in the morning ewes received 200 g soybean animal/day.

Five female pluriparous Santa Ines (average age of 3 years), that were non-lactating, clinically healthy and recorded a body condition score (BCS) of 3.0 ± 0.3, were used as oocyte donors. Similarly 10 multiparous clinically healthy Santa Ines ewes, that also recorded a BCS of 3.0 ± 0.3, were used as embryo recipients.

Based on the results of a preliminary study (data not shown) and in accordance with Baldassarre et al. (2007), all oocyte donors were treated with a one-shot hormonal regime (Baldassarre et al., 1996). In this protocol, estrus was synchronized by means of intravaginal devices containing 0.3 mg progesterone (CIDR®, Pfizer, Netherlands) maintained from D_0 to D_{10}. On D_8 donors were injected intramuscularly with 0.04 mg sodium D-cloprostenol (Ciosin®, Coopers, Brasil), 200 IU FSH (Folltropin®, Bioniche, Canada) and 300 IU eCG (Novormon®, Syntex, Argentina), as set out in Fig. 1 The recipients were synchronized with subcutaneous implants inserted in the ear containing 1.5 mg Norgestomet (Crestar®, Intervet, Holland) on D_0, maintained for 9 days. At progesterone implant removal, recipients received 400 IU eCG (Folligon®, Intervet, Netherlands) and 0.04 mg D-cloprostenol sodium (Ciosin®, Coopers, Brasil), as schematically illustrated in Fig. 2.

Follicular puncture for oocyte aspiration of the ovaries was performed via surgical mid-ventral laparotomy, using a disposable hypodermic 21 gauge, 30 × 8 mm needle (Becton Dickson, Brazil), connected to a 50 ml conical tube (Corning, USA), with a 0.8 m silicone tube (internal diameter of 2 mm). Aspiration was performed using negative pressure (vacuum), corresponding to a flow of 13 ml H_2O/min, as described by Seneda et al. (2001).

The medium used for aspiration was composed of a PBS solution, plus 0.05 mg/mL heparin. All the visible follicles on both ovaries of each ewe were counted and aspirated. The aspirated fluid was then evaluated under a stereomicroscope (Meiji Techno EMZ, Japan), and the oocytes classified according to Seneda et al. (2001).

All classified oocytes were placed into cryovials containing TCM 199 (Nutricell, Brazil) and mineral oil, previously stabilized in an incubator at

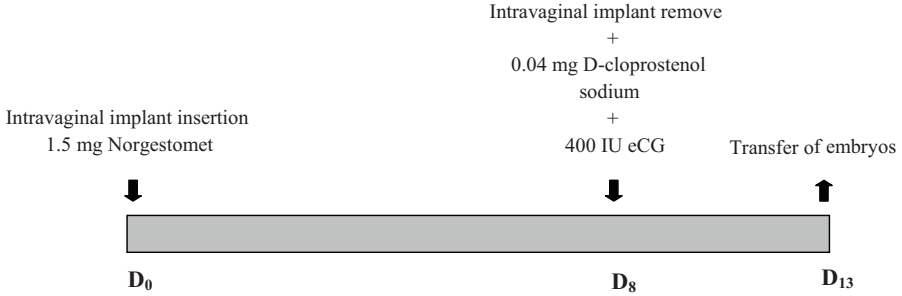

Fig. 2. Schematic presentation of the protocol for synchronization of recipient ewes.

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Table 1
The mean (±SD) ovine oocytes retrieved by follicular aspiration after laparotomy and embryos, generated following IVEP after long distance transport.

Classification of oocytes and embryos	Mean oocytes ± SD (total oocytes/donor)	Mean embryos ± SD (total embryos/donor)
Grade I	4.6 ± 2.9 (23/5)	5.4 ± 3.1 (27/5)
Grade II	1.6 ± 0.9 (8/5)	2.4 ± 1.6 (12/5)
Grade III	1.8 ± 0.8 (9/5)	–
Denuded	1.0 ± 1.7 (5/5)	–
Degenerated	0.8 ± 0.8 (4/5)	2 ± 2 (10/5)
Mean ± SD/Total	9.8 ± 5.7 (49/5)	9.8 ± 3 (49/5)

38.5 °C, and 5% CO_2. Before placing the material in the transport incubator (TO-16, WTA, Cravinhos, Sao Paulo, Brazil), the cryovials were flushed with CO_2, sealed, and wrapped with parafilm. The transport incubator maintained an environment of 38.5 °C and 5% CO_2 and had a battery life of 24 h. This system for in vitro maturation (IVM) of oocytes following transport was previously been described for bovine (Pontes et al., 2010)

In the laboratory, the oocytes were re-evaluated and classified, after completing 24 h of in vitro maturation (IVM) – initially 14 oocytes while transported, and finally 10 in the laboratory. All oocytes were fertilized with frozen semen from a single Santa Ines ram. The sperm sample was prepared using the Percoll gradient (Pontes et al., 2009). The fertilization was performed in IVF-TALP for 18 h, and this was followed by in vitro culture (IVC) in SOFovine (In Vitro Brasil, Sao Paulo, Brazil). After a 24 h period culture, the embryos were placed into cryovials containing the same culture medium, under mineral oil. The cryovials were all properly identified and placed in the same portable incubator at 38.5 °C and 5% CO_2 and returned to Londrina, Parana State, under the same conditions (time and distance). Upon arrival at the farm, the cryovials were aerated with CO_2 every 12 h, until transfer of the embryos.

The embryos were transferred to the ewe recipients that had previously been synchronized, according to the hormonal protocol (Fig. 2). A mid-ventral laparotomy was then performed on the 10 synchronized recipients, but only those with at least one corpus luteum ($n = 7$), received the embryos. Prior to transfer the embryos were evaluated under a stereomicroscope (Meiji Techno EMZ, Japan) and packaged in 0.25 mL straws (3 or 4 embryos per straw). The transfer was performed at the apex of the uterine horn, ipsilateral to the corpus luteum. The incision being made with the aid of a micropipette tip (yellow 100 μl tip). Pregnancy diagnoses were performed using a transrectal ultrasound, 30 days after embryo transfer.

3. Results

The aspiration of the 5 donors resulted in 49 oocytes, approximately 10 oocytes per donor of varying quality (Table 1). In vitro fertilization (IVF) was performed on all collected oocytes, and embryos being at the morula stage on day 4 of incubation (Table 1). All the oocytes ($n = 49$) of grades I, II, III or denuded had successfully cleaved and 25 embryos were transferred into the recipients. The number of recipients available was lower than the number of embryos produced, thus only 25 embryos were transferred into the recipients that exhibited 1–4 corpora lutea (CLs). One pregnancy (14.3%) was confirmed. A healthy lamb was born after normal delivery.

4. Discussion

This study demonstrated the possibility of in vitro oocyte maturation and in vitro embryonic development using a transportable incubator. To current knowledge, this is the first report describing this methodology in sheep, which could be very useful in situations where in vitro laboratories are distant from the farms.

The average collection yield of 9.8 ± 5.7 oocytes per donor using the one-shot hormonal regime was comparable to the results reported by Baldassarre et al. (1996) in sheep of a different breed in Argentina. However lower than that reported by Baldassarre and Karatzas (2004), who obtained an average of 13.4 oocytes per donor in goats in Canada. In Brazil, Basso et al. (2008) reported an average of 10 oocytes per donor for Santa Ines ewes, which is consistent with the present results. In the literature, a comparison in the production of oocytes between different breeds of sheep has not been reported, but it is believed that there may be significant differences in this regard.

In the current work, laparotomy was chosen as the technique for oocyte collection and the transfer of embryos. Laparoscopy as such, has been described as the best method for follicular aspiration, as it induces minimal stress to the animal and allows for repeated sessions of follicular aspiration – with little risk of loss in future fertility of the donor, due to its low invasiveness, compared to laparotomy (Baldassarre et al., 1996; Stangl et al., 1999). However, this technique of laparoscopy presents some disadvantages, such as the high cost of the equipment and the training that is needed for both the operator and helper to effectively minimize trauma and surgical time (Tabet, 2007). In this respect, laparotomy may be considered as an alternative method. Li et al. (2008) showed no significant difference in the pregnancy rates when either laparoscopy or laparotomy was used for the transfer of embryos that were produced in vitro.

In the present trial, different qualities of oocytes were obtained, and all were sent to the laboratory for IVF, except those oocytes graded as degenerated. All cleaved oocytes then reached the morula stage by the fourth day after fertilization.

Obtaining a successful pregnancy and a viable lamb as a result of this work, has highlighted that the transport of oocytes and embryos in specific environments for long periods of time (over long distances) is a viable alternative that can be used to minimize the geographic barriers between laboratories and field practitioners, also to overcome difficulties associated with cryopreservation of ovine embryos that are produced in vitro.

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