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First llama (*Lama glama*) pregnancy obtained after *in vitro* fertilization and *in vitro* culture of gametes from live animals

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ABSTRACT

The aim of this study was to evaluate the developmental competence and pregnancy rate of llama hatched blastocysts produced *in vitro* using gametes from live animals and two different culture conditions. Fifteen adult females were superstimulated with 1500 IU of eCG, eleven (73%) responded to the treatment and were used as oocyte donors. Follicular aspiration was conducted by flank laparotomy. Semen collections were performed under general anesthesia by electroejaculation of the male. Sixty-six COCs were recovered from 77 aspirated follicles (86% recovery) and were randomly placed in Fertil-TALP microdroplets with the sperm suspension (20×10^6 live spermatozoa/ml). After 24 h, they were placed in SOFaa medium supplemented with FCS and randomly assigned to one of two culture conditions. Culture condition 1 (CC1) consisted of 6 days of culture ($n = 28$) and culture condition 2 (CC2) consisted of renewing the culture medium every 48 h ($n = 35$). In CC1, the blastocyst rate was 36% (10/28) and the hatched blastocyst rate was 28% (8/28) whereas in CC2, the blastocyst rate was 34% (12/35) and the hatched blastocyst rate was 20% (7/35) ($p > 0.05$). No pregnancies were obtained after embryo transfer (ET) of CC1 blastocysts (0/8) while one pregnancy was obtained (1/7) after transferring a hatched blastocyst from CC2. Forty-two days after the ET, the pregnancy was lost.

This study represents the first report of a pregnancy in the llama after intrauterine transfer of embryos produced by *in vitro* fertilization using gametes from live animals.

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1. Introduction

Over the last few years, an increasing interest in the production of South American Camelids (SAC) has been developed, not only in South America but also in different countries around the world. Because these species present a long period of gestation (335–360 days; Johnson, 1989; Leon et al., 1990) and only deliver one young per year, it is of interest to apply assisted reproductive techniques to optimize the reproductive handling of genetically

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superior females and to increase the genetic progress of these species. The final objective of *in vitro* embryo production is to develop high quality embryos and obtain normal pregnancies after transfer to recipient females, which finally result in the birth of healthy offsprings, a goal not yet attained in SAC.

There are few reports published on *in vitro* fertilization (IVF) in SAC. The first IVF in llamas was carried out by Del Campo et al. (1994). Out of the 234 zygotes cultured using epithelial oviduct cell co-culture, only 4.7% (11/234) developed to the hatched blastocyst stage and no embryo transfers were reported in this study. Gomez et al. (2002) reported the first production of llama-alpaca crossbreed embryos after heterologous IVF; after 6 days of culture all fertilized oocytes reached the morula stage ($n = 5$), but none of them continued *in vitro* development. Both of these studies worked with gametes from slaughterhouse animals but it's important to apply this kind of technology in live animals. Besides, the development of a simple and viable culture system to reach embryo growth beyond the morula stage after IVF is vital for implementing an intrauterine embryo transfer (ET) program. We have recently reported the first production of *in vitro* llama embryos that developed to the hatched blastocyst stage. These were obtained after IVF using spermatozoa selected with Androcoll-E™ from raw semen, oocytes from superstimulated females and *in vitro* culture in synthetic oviduct fluid medium with amino acids (SOFaa) and with bovine serum albumin (BSA) during 6 days (Trasorras et al., 2012). In dromedary, offspring were obtained from *in vitro* produced embryos achieved after adding fetal calf serum (FCS) to the embryo culture medium and reaching the hatched blastocyst stage (Khatir and Anouassi, 2006).

When an embryo culture system is developed, the amount of time that embryos will be in contact with the medium is an important factor to take into account. The culture medium should not be considered a static system; the embryos themselves alter its composition, especially when amino acids are added. Studies carried out in humans (Virant-Klun et al., 2006), mice (Gardner and Lane, 1993) and sheep (Gardner et al., 1994) for *in vitro* culture embryo development to the blastocyst stage, have demonstrated that although the addition of amino acids to the culture medium had a significant effect on both embryo cleavage rate and morphological development, the beneficial effects on cleavage decreased in relation to the duration of culture. It was determined that amino acids are both metabolized by embryos and spontaneously broken down at 37 °C, thus producing significant levels of ammonium in the medium. The ammonium generated from the amino acids was found to not only inhibit cleavage and blastocyst development (human: Virant-Klun et al., 2006; mouse: Gardner and Lane, 1993) but also to be associated with subsequent fetal retardation and neural tube defects in mice (Lane and Gardner, 1994). In sheep, increased embryo cleavage and development rates in culture, in the presence of amino acids, were obtained by placing embryos in fresh medium every 48 h to alleviate the toxic effects of ammonium (Gardner et al., 1994).

The aim of this study was to evaluate the developmental competence and pregnancy rate of llama hatched blastocysts produced *in vitro* using gametes from live animals and two different culture conditions.

2. Materials and methods

2.1. Animals

Thirty non-pregnant, non-lactating female llamas, ranging between 4 and 8 years of age and with an average body weight of 120 ± 22 kg were used in this study. Of the 30 females, 15 were used as oocyte donors and 15 as ET recipients. All females were kept separate from the males during the experiment and fed with hay and water *ad libitum*. The study was conducted at the Faculty of Veterinary Sciences of the University of Buenos Aires, Buenos Aires, Argentina, situated 34°36′ S and 58°26′ W, at sea level. This study was approved by the Committee for the Use and Care of Laboratory Animals (CICUAL) of the Faculty of Veterinary Sciences of the University of Buenos Aires (protocol no. 2010/24).

All reagents were purchased from Sigma (St. Louis, MO, USA) except where stated otherwise.

2.2. In vivo oocyte recovery

2.2.1. Management of the oocyte donor females

Ovarian dynamics were monitored by transrectal palpation and ultrasonography (Berger LC 2010 plus with a 5 MHz linear-array electronic transducer, Buenos Aires, Argentina). The absence of follicles larger than 5 mm was confirmed before beginning the superstimulation treatment and a single IM dose (1500 IU) of eCG (Novormon®, Syntex, Argentina) was administered ($n = 15$) (Trasorras et al., 2009). Positive response to eCG treatment was considered when a female presented, in each ovary, two or more follicles $\geq$7 mm (dominant follicle) at ultrasound evaluation.

2.2.2. LH surge induction

Five days after the superstimulatory treatment, females with a positive response to eCG were selected for follicle aspiration and received a single IV dose of 8 μg of buserelin (Receptal®, Intervet, Buenos Aires, Argentina) for *in vivo* oocyte maturation within the follicles. Twenty hours later, females were subjected to surgical procedures and follicular aspiration.

2.2.3. Surgical procedures and oocyte evaluation

Females selected for surgery were deprived of solid food 24 h and water 18 h previously. The technique was performed as previously described (Trasorras et al., 2009). Briefly, general anesthesia was induced by IV administration of 0.2 mg/kg of xylazine (Rompun®, Bayer, Buenos Aires, Argentina), 1.5 mg/kg of ketamine hydrochloride (Ketamina®, Holliday, Buenos Aires, Argentina) and 0.1 mg/kg of butorphanol (Torbutrol plus®, Fort Dodge, La Plata, Argentina). Local anesthesia of the surgical area was carried out using 2% lidocaine (Equi Systems®, Buenos Aires, Argentina); general anesthesia was maintained by intravenously injecting half the induction dose of ketamine and xylazine, as needed. The superstimulated ovaries were exposed, with transrectal manual aid, through an 8–10 cm

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