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Use of IVF and ET in Mexican Criollo Sheep (*Ovis aries*): Immediate and Delayed Embryo Transfers

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Abstract

There is little information in the scientific literature concerning sheep pregnancy and lambing success with regard to the timeframe from when *in vitro* produced embryos are transported to the designated location for embryo transfer (ET). The aim of this study was to transfer *in vitro* produced embryos under two different conditions that could typically occur using the aforementioned assisted reproductive techniques (ARTs). Abattoir ovaries were used to procure oocytes for *in vitro* embryo production and subsequent transfer to synchronized ewes. The study consisted of two experiments: Experiment 1 (Exp1)—embryos taken from the laboratory to a nearby surgical room for immediate ET, and Experiment 2 (Exp2)—ET after 5 hours (h) of transport to a rural farm. Lambing in relation to detected pregnancies, births compared to pregnancies, and the proportion of twin offspring were all higher in Exp2. Notably, in both Exp1 and Exp2, there was not a significant difference ($P > 0.05$) between the number of embryos transferred, *i.e.*, 3 versus 4, respectively, and the number of ewes that underwent parturition in each group. Also, in both experiments there was not a significant difference ($P > 0.05$) in the number of ewes that underwent

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parturition based on the number (*i.e.*, ≥1) of corpora lutea present. The results of the present study demonstrate the importance of evaluating different conditions when applying ARTs, as there are many variables that can influence the outcome. Importantly, Exp2 results show that ovine ET in places located far away from the embryo production site can be useful and successful provided that embryo transport, ET, and recipient conditions are adequate.

Keywords

Embryo (Transfer), Fertilization (*In Vitro*), Ovine, Reproduction (Assisted), Sheep

1. Introduction

In vitro embryo production is possible from follicular immature oocytes that can be matured and fertilized in the laboratory and cultured until the morula and blastocyst stages. Subsequently, the embryos can be transferred to synchronized recipient females to develop offspring [1]. The aforementioned procedure has a great impact worldwide by increasing the number of animals possessing high-quality genetic characteristics, whether domestic, wild or endangered species, and even for assisted reproduction of humans [2] [3].

Embryo production from immature oocytes developed *in vitro* can offer several possibilities; such as, using ovaries from slaughtered females of seasonal mammalian species like sheep, increasing animal production, and using sperm from selected sires of the highest genetic value [4] [5]. The use of immature oocytes for embryo production also allows for the study of basic, molecular, genetic, physiological, and cellular aspects of embryo development, cloning, and transgenesis [3] [6]-[8].

For assisted ovine reproduction, embryos are obtained from superovulated and artificially inseminated females and then transferred to recipients; however this procedure is expensive, and only a limited number of embryos can be processed [9] [10]. In comparison, embryos can be produced using larger quantities of oocytes through *in vitro* maturation (IVM) and *in vitro* fertilization (IVF), resulting in more embryos to be transferred to recipients [11].

In recent years, several methods using defined media have been performed to improve assisted reproduction. However, it is important to realize that the conditions for embryo transfer (ET) may vary due to different situations such as breed, age, nutrition, body condition, and semen quality, which may have an effect on pregnancy success and the birth of healthy animals [1] [3] [9]. Another important factor of ET is embryo transport time and transport conditions from the IVF laboratory to the place where the embryos will be transferred to recipients [2] [12].

One inconvenience for the application of this assisted reproduction methodology is the distance between the laboratory where the embryos are produced and the place for ET. Ideally, the embryos should be transported maintaining the same conditions as in the laboratory [2]. In the case of sheep, there are reports indicating that follicular oocytes obtained far away from the laboratory have been transported at 38.5˚C for 14 h for IVM, IVF and *in vitro* production (IVP). Max *et al.* [13] reported that after three days of development, the embryos are returned back to the same place where the oocytes were obtained, and 30 days (d) after ET, one recipient was pregnant. It is beneficial to both the producer and consumer to increase the knowledge about the efficiency of assisted reproductive techniques (ARTs) under alternative conditions, as logistics in certain localities may be more complex than others and this may affect the success of pregnancy and lambing rates.

The objective of the present research was to transfer *in vitro* produced Mexican Criollo sheep embryos in order to evaluate the efficiency of this methodology for reproductive programs and under alternative conditions. A statistical comparison between the two experiments was not made (since they were conducted under different conditions); however, statistical analyses of the results of each independent experiment were performed.

2. Material and Methods

2.1. Oocyte Collection, IVM and IVF

Unless otherwise stated, all chemicals were purchased from Sigma® (St. Louis MO, USA). Ovaries were col-

lected from Mexican Criollo ewes (*Ovis aries*) at the slaughterhouse "El Rojo" (Estado de Mexico), placed in a 9% NaCl solution containing 10,000 UI·mL^{-1} penicillin, 10 mg·mL^{-1} streptomycin sulfate salt and 25 µg·mL^{-1} amphotericin, and transported (<2 h) to the laboratory "Manejo de la Reproducción" at the Universidad Autonoma Metropolitana Unidad Xochimilco (Mexico). The ovaries were washed 3 times with the aforementioned solution. Afterwards, the ovary follicles of 2 to 8 mm in diameter were aspirated. The follicular fluid was collected with an 18-ga needle fixed to a 10-mL disposable syringe containing 1 mL of modified Tyrode's medium supplemented with 10 mM sodium lactate, 0.50 mM HEPES, 0.01% polyvinyl alcohol [14] and 5 U.I. heparin·mL^{-1} [15]. Oocytes surrounded with at least four layers of compact cumulus cells and uniform cytoplasm were selected [16] and washed three times in 500 µL drops of protein-free maturation medium TCM 199 with Earle's salts and 26.2 mM sodium bicarbonate (*in Vitro*, Mexico) supplemented with 0.1% polyvinyl alcohol, 3.05 mM D-glucose, 0.91 mM sodium pyruvate, 0.57 mM cysteine, 10 ng·ml^{-1} epidermal growth factor, 0.5 µg·mL^{-1} LH, and 0.5 µg·mL^{-1} FSH [17] covered with mineral oil (Fisher Scientific, USA). Fifty oocytes were placed in each well of a four-well dish (Nunc, Denmark) containing 500 µL of maturation medium and incubated at 38.5˚C with 5% CO_2 in air and humidity at saturation for 24 h [18].

After IVM, oocyte-cumulus cell complexes were washed three times in 100 µL drops of modified Tris-buffered medium (mTBM) consisting of 113.1 mM NaCl, 3 mM KCl, 7.5 mM CaCl2·2H_2O, 20 mM Tris, 11 mM glucose, 5 mM sodium pyruvate, supplemented with 0.4% bovine serum albumin fraction V, and 2.5 mM caffeine. The aforementioned medium was also used for IVF [17]. Twenty oocytes were placed in each well of a four-well dish in 250 µL of mTBM, covered with mineral oil, and incubated for 30 minutes (min) until insemination. For oocyte insemination, frozen-thawed semen from a fertile ram was used. In a water bath, two 0.5 mL straws containing 100 × 10^6 sperm were thawed for 45 seconds (sec) at 37˚C [19]. Sperm were diluted 1:3 with mTBM and centrifuged at 1000 x g for 4 min at 22˚C - 25˚C. The sperm pellet was diluted with 100 µL of TCM 199-H, and 2 mL of the same medium was added to a 45˚ bent tube for 30 min swim-up [20]. The supernatant was diluted in the same medium to give a concentration of 1 × 10^6 cells·mL^{-1}, and 250 µL were added to each well [21] for a final concentration of 5 × 10^5 cells·mL^{-1}. The gametes were co-incubated for 18 h under the previously described conditions [22].

2.2. Embryo Production

After the co-incubation period, cumulus cells were removed and putative zygotes were transferred to each well containing 500 µL of synthetic oviductal fluid developmental medium, containing 107.70 mM NaCl, 7.16 mM KCl, 1.19 mM KH_2Po_4, 1.71 mM $CaCl_2$, 0.49 mM $MgCl_2$, 25.07 mM $NaHCO_3$, 3.30 mM Na lactate, 0.33 mM Na pyruvate, 1.50 mM glucose, 32 mg/mL bovine serum albumin, 100 unit penicillin (sodium salt)/mL and 50 µg streptomycin/mL [22] [23], supplemented with 32 mg·mL^{-1} bovine serum albumin fraction V [24] and 10% inactivated fetal bovine serum [25], and incubated for 5 d at 38.5˚C, 5% CO_2 in air and 90% relative humidity. The embryo stage was evaluated according to the International Embryo Transfer Society guidelines [26].

2.3. Embryo Transfer

For ET, ewes were synchronized with sponges impregnated with 20 mg Chronolone (Chronogest CR, Intervet) for 12 d. At the time of sponge removal, 500 IU eCG were injected IM (Folligon, Intervet) [12]. Twenty-four h after sponge removal, estrus was detected by exposing the ewes to an adult male fitted with an apron every 12 h over a period of 2 d.

Embryos were transferred to recipient females by abdominal laparoscopy. Food and water were suspended to the recipients 48 h before surgery for all of the recipients. For general anesthesia, ewes were injected with 0.2 mg·kg^{-1} Xylazine IM and 5 mg·kg^{-1} Ketamine hydrochloride IV [27]. Subsequently, local anesthesia was applied with 2% Xylocaine SC. Laparoscopy was performed according to Li *et al.* [15]. The uterine horn ipsilateral to the ovary with the largest number of corpora lutea was selected and punctured with a blunt tipped 18-ga needle to deposit the embryos in the lumen with an IVF catheter. Four embryos in morula or blastocyst stage were transferred to each ewe. Afterwards, the uterine horn was returned to the abdominal cavity and the muscular fascia and skin were sutured. Pregnancy diagnosis was performed by ultrasound (Esaote Pie Medical, Tringa Linear) using a 5 MHz linear probe, 24 d after ET [28]. The number of embryos, pregnancy rates and lengths,

births, as well as birth weights were recorded.

2.4. Statistical Analysis

For each independent experiment, statistical analyses with regard to the number of ewes producing lambs based on the number of embryos transferred and the number of corpora lutea present were conducted using the Fisher's exact test. Statistical differences were considered to exist at $P < 0.05$ (*i.e.*, Type I error was set at $\alpha = 0.05$).

2.5. Study Design

To evaluate surgical ET efficiency under different scenarios, the study consisted of two experiments: Experiment 1 (Exp1)—ET in less than 10 min of transport from the laboratory to a nearby surgical room. Experiment 2 (Exp2)—ET after 5 h of transport from the laboratory to a surgical room located in a rural sheep production farm.

For Exp1, six crossbreed multiparous females were fed alfalfa hay (IFN 2-00-81) and a commercial concentrate (Ovina 14, Purina, Mexico). Embryos were transported from the laboratory to the surgical room in holding medium (Vigro, Bioniche, Canada) and incubated at 37.5˚C in an IVF catheter until transfer.

For Exp2, ten crossbreed nulliparous females were fed corn silage (IFN 3-02-912), oat hay (IFN 1-03-280) and a commercial concentrate (Ovina 14, Purina, Mexico). Embryos were transported to the surgical room of a sheep production farm in 0.25 mL plastic straws in the same holding medium at 37.5˚C. The surgical ET procedure was the same in both experiments.

3. Results

3.1. Experiment 1

Five days after IVF, different stages of embryo development were identified and recorded. A total of sixteen morulae and five blastocysts (three or four embryos/ewe) were transferred to recipients. At day 24 after ET, pregnancy was determined via ultrasound and four of six ewes were pregnant. From the three ewes that received four embryos, three fetuses were detected in one of the ewes; another ewe had two fetuses; and the remaining ewe was not pregnant. From the three ewes receiving three embryos, two fetuses were detected in two of them, and one was not pregnant. The gestation period ranged from 148 to 151 d (**Table 1**). From the four pregnant recipients, the number of delivered lambs was lower than the number of transferred embryos. Five lambs were delivered, two males and three females. Birth weights were from three to five kg with a birth rate of 125% because one female delivered twins (**Table 2**).

In Exp1 there was not a significant difference (two-tailed $P = 1$) in the number of ewes receiving three versus four embryos and the number of ewes that underwent parturition. Also, in this experiment there was not a significant difference (two-tailed $P = 0.3333$) in the number of ewes giving birth to lambs with regard to the number of CLs present, specifically, between those ewes with one CL versus more than one CL.

3.2. Experiment 2

Five days after IVF, different stages of embryo development were identified and recorded. A total of 35 morulae and 2 blastocysts (three or four embryos/ewe) were transferred after 5 h of transport. Twenty-four days after ET, pregnancy was determined and five out of ten ewes were pregnant. Of the seven ewes receiving four embryos, two had two fetuses; another had only one fetus; and the four remaining ewes were not pregnant. Of the three ewes receiving three embryos, one had two fetuses, another had only one fetus, and the last was not pregnant. The gestation period ranged from 151 to 158 d. Eight lambs were delivered, three males and five females. The birth weights were from 2.8 to 3.5 kg (**Table 3**) with a birth rate of 160% due to the fact that three females delivered twins (**Table 2**).

In Exp2, there was not a significant difference (two-tailed $P = 1$) in the number of ewes receiving receiving three versus four embryos and the number of ewes that underwent parturition. Also, in this experiment there was not a significant difference (two-tailed $P = 1$) in the number of ewes giving birth to lambs with regard to the number of CLs present.

Table 1. Embryo transfer (ET), pregnancy and lambing in Experiment 1.

Ewe	*Corpora lutea*	*ET* (*morulae* + *blastocysts*)	*Detected fetuses*[a]	*Gestation period* (*days*)	*Delivered lambs*	*Birth weight* (*kg*)
1	5	4	3	151	2	3; 5
2	1	3	0	NP	0	0
3	2	3	2	151	1	4
4	2	3	2	149	1	5
5	2	4	0	NP	0	0
6	2	4	2	148	1	3

NP = Not pregnant; All embryos were transferred to the right uterine horn ipsilateral to corpora lutea; a. Fetus detection by ultrasound 24 days after ET.

Table 2. Embryo transfer (ET), pregnancy and lambing efficiency in Experiments 1 and 2.

	Experiment 1 (%)	Experiment 2 (%)
Detected fetuses[a]/ET	9/21 (43)	8/37 (22)
Births/ET	5/21 (24)	8/37 (22)
Births/Detected fetuses[a]	5/9 (56)	8/8 (100)
Pregnant/Total females	4/6 (67)	5/10 (50)
Births/Pregnant females	5/4 (125)[b]	8/5 (160)[c]

a. Fetus detection by ultrasound 24 days after ET; b. Three ewes delivered twins; c. Three ewes delivered twins

Table 3. Embryo transfer (ET), pregnancy and lambing in Experiment 2.

Ewe	*Corpora lutea*	*ET* (*morulae* + *blastocysts*)	*Detected fetuses*[a]	*Gestation period* (*days*)	*Delivered lambs*	*Birth weight* (*kg*)
1	2	4	2	155	2	2.8; 3.2
2	1	4	0	NP	0	0
3	2	4	1	153	1	3.5
4	2	4	0	NP	0	0
5	3	4	0	NP	0	0
6	3	3	2	152	2	2.8; 3.5
7	2	4	0	NP	0	0
8	2	3	0	NP	0	0
9	2	4	2	151	2	3; 3.4
10	1	3	1	158	1	3.4

NP = Not pregnant; All embryos were transferred to the right uterine horn ipsilateral to corpora lutea; a. Fetus detection by ultrasound 24 days after ET.

4. Discussion

Assisted reproduction specialists require the development of better techniques to increase the reproductive capacity and genetic improvement of many domestic and wild animal species. Development and application of IVP and ET techniques in livestock, e.g., domestic sheep, may contribute to multiply selected populations with exploitable characteristics. ARTs such as IVP and ET can help produce larger numbers of individuals at a faster

rate than normal, and selected according to size, meat, and milk or wool production. The ARTs can also assist in the development of new breeds by facilitating the crossbreeding of genetically selected animals that may be better adapted to certain environments. IVP provides an excellent source of low cost material for basic research on developmental biology and physiology, and has commercial application in some biotechnologies such as nuclear transfer and transgenesis [6] [7]. However, research is still required in order to improve the efficiency of these techniques, thereby making all of these procedures more competitive and profitable. The embryo production laboratory, in some cases, is far away from the sheep production farms. Therefore, it is important to preserve embryo quality in order to obtain acceptable pregnancy rates.

The percentage of pregnant females out of the total number of recipients was somewhat higher in Exp1 (**Table 2**). In Exp1, the percentage of fetuses detected by ultrasound per the number of transferred embryos was about twofold that of Exp2. However, the percentage of births per the total number of transferred embryos was similar in both experiments. The percentage of births per pregnant ewe was higher in Exp2. The differences between the experiments demonstrate the importance of not relying upon only one set of conditions for determining the potential outcome of applying ARTs. The differences between the two experiments could be due to the transport period (5 h), thereby reducing the embryo's ability to develop an initial pregnancy.

Importantly, the results of the two experiments cannot be compared against one another statistically given that there were differences in locations, dietary conditions, and environmental conditions; therefore, the results of each experiment were analyzed individually. The statistical analyses revealed that there was not a significant difference ($P > 0.5$) in the number of ewes giving birth to lambs with regard to the transfer of three versus four embryos in either of the experiments. Further, there was not a significant difference in either of the experiments with regard to the number of CLs present, specifically, between those ewes having one CL versus more than one CL, and the number of ewes giving birth to lambs.

The results of the present study are similar to those reported by Papadopoulos *et al.* [29] who obtained a 32.8% embryo survival out of the number of blastocysts transferred. When analyzing the birth proportion regarding the quantity of detected fetuses and the number of pregnant females, it was observed that pregnancy outcome in Exp2 was about twofold that of Exp1. Also, the efficiency of births over pregnancies was higher in Exp2 as well. Overall, these results indicate that once the pregnancy is established, other conditions are involved in pregnancy outcome, e.g., the stress-inducing reduced space in the Exp1 animal facilities, in comparison to the larger space conditions at the farm of Exp2, as well as female characteristics such as breed and feed (*i.e.* nutritional components) occurring in Exp2. The pregnant female proportion in Exp1 and Exp2 (67% and 50%) respectively, as well as the number of pregnant females, are within the range reported by other authors [10] [12] [15] [30]. In Exp1 the gestation length period ranged from 148 to 151 d, compared to Exp2 ranging from 151 to 158 d. This study showed that the gestation period can vary under different sets of conditions, e.g., longer in Exp2 than in Exp1. The results of the present research show lower pregnancy lengths than those reported by Holm *et al*. [1] in Merino females (154 - 155 d) using embryos obtained *in vitro*. The pregnancy rate in both experiments, 67% (Exp1) and 50% (Exp2), was higher than that obtained by Li *et al*. [15] who crossbred Merino males to Dorset females, obtaining a 47.6% pregnancy rate.

It is important to use multiparous females in order to get higher pregnancy rates [15]. In the present study, the recipients were from one (Exp2) to 3.5 (Exp1) years old; and were nulliparous (Exp2) and multiparous (Exp1). Another important factor in the pregnancy outcome could be nutrition. In some cases recipients were fed a balanced diet consisting of a mix of alfalfa hay, corn silage, and commercial concentrate [15], but others were fed only grass [10]. The Exp1 recipients were fed alfalfa hay and a commercial concentrate. The lower proportion of lambs obtained in contrast to Exp2 could in part be influenced by a diet with high protein content. It is known that urea can affect protein metabolism and therefore may affect survival of sheep embryos. Ewes fed with higher nitrogen levels degraded it in the rumen thereby increasing urea and ammonia concentrations in plasma and the uterus, which could be associated with embryo death [9].

Regarding birth weight, Exp1 lambs tended to be heavier (3 to 5 kg) than the lambs of Exp2 (2.8 to 3.5 kg). Other studies using IVP sheep embryos transferred to recipients showed similar birth weights. Holm *et al*. [1] reported lamb weights 4.5 to 5.1 kg, and Morton *et al.* [10] 3.8 kg. Notably, only 2 of the 13 lambs in the present study had a weight of 5 kg, the average of Exp1 and Exp2 was 4 and 3.2 kg, respectively. The results of the present research are similar with regard to birth weights (3.1 to 5.4 kg) of lambs from ewes after artificial insemination, indicating that the large birth weight frequently observed after ET of IVP embryos [31] was not an issue.

In Exp1, five lambs were delivered from four ewes, *i.e.*, birth rate of 125%, as one female delivered twins. In

Exp2, eight offspring were born from five ewes, *i.e.*, birth rate of 160%, due to three females delivering twins. Interestingly, the proportion of twin deliveries in Exp2 was more than twice that of Exp1.

5. Conclusions

As mentioned in Seidel [32], recent methods of estrus synchronization in cattle and sheep do not require detection of estrus for fixed time embryo transfer (ET). Therefore, embryo transfer can now be carried out easily both in the clinic and on the farm, and this can help solve the limitation of not having enough recipients available when IVF-derived embryos are ready to be taken out of the incubator for ET. As is currently done in cattle IVF embryo production, a larger number of sheep embryos can be produced, prepared, and shipped for transfer at different times and places, thereby increasing embryo and recipient use.

This study contributes to the ART literature by increasing the knowledge of sheep IVF and ET, as well as its use in situations similar to those commonly seen in cattle, in which the donors and recipients are located in separate locations. More specifically, the results of Exp2 show that ET in places located far away from the embryo production site can be useful and successful provided that embryo transport, ET, and recipient conditions are adequate. Given the differences in the results of Exp1 and Exp2, a future study with a larger number of animals should be conducted to determine which factor is mainly responsible.

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