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Pregnancy Rate Following Transfer of *in vitro* Produced Lamb Derived Embryos in Two Embryonic Stages

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Abstract: Ovine embryos were produced by maturation, fertilization and *in vitro* culture (IVM/IVF/IVC) of oocytes collected from slaughtered prepubertal ewes. At 24 h post IVM, oocytes were fertilized with fresh semen collected from Lori-Bakhtiari breed at a concentration of 1.0×10^6 sperm mL^{-1} . The presumptive ova/embryos were transferred into the embryo culture medium at 22-24 h post IVF. Following 4 to 7 day in culture, embryos (at morula and blastocyst stage, respectively) were transferred surgically to the uterine horn of synchronized recipients. Pregnancy was diagnosed at day 30 by hormonal assay and at days 55 and 140 of gestation by ultrasonography and pregnancies were allowed to go to term. A total of nine ewes received 27 embryos (3 embryos/ewe). Five ewes received 15 embryos at morula stage and four ewes received 12 embryos at blastocyst stage. From those received morula stage embryos one was pregnant on day 30 (20%), though no pregnancy was diagnosed on each of days 55 and 140. While from those received blastocyst stage embryos, three ewes were pregnant on day 30 (75%) and two ewes (50%) remained pregnant on each of days 55 and 140. In conclusion, day 4 IVM-IVF morula stage embryos had a lower survival rate than did day 7 IVM-IVF blastocysts embryos, following transfer to the synchronized recipient ewes.

Key words: Sheep, morula, blastocyst, *in vitro* fertilization

INTRODUCTION

It is documented that the pregnancy rate following transfer of fresh *in vitro* produced (IVP) blastocysts is lower than that of *in vivo* embryos (54.3% versus 90.0%, respectively; Papadopoulos *et al.*, 2002). Pregnancy rates after fresh embryo transfer, produced in *in vitro* condition, varied from 29 to 69% in sheep (Slavik *et al.*, 1992; Thompson *et al.*, 1995; Brown and Radziewicz, 1998).

The poorly defined culture systems used in early attempts to produce ruminant embryos *in vitro*, resulted in embryos with clear morphological differences compared to *in vivo*-produced counterparts (Dorland *et al.*, 1994; Thompson *et al.*, 1995). The offspring generated by transfer of such embryos were subject to a variety of developmental defects and decreased the efficiency of such methods (Behboodi *et al.*, 1995; Sinclair *et al.*, 1998). In the last decade, substantial progress in terms of morphology of IVP ruminant embryos has been achieved using defined and semi-defined culture systems (Thompson *et al.*, 1995; Bernardi *et al.*, 1996). Accordingly, in recent studies the embryonic loss following transfer of IVP ruminant embryos has been substantially decreased and there was no evidence of

congenital defects, enhanced gestation length, or prenatal loss (Thompson *et al.*, 1995; Ptak *et al.*, 2002).

Despite all efforts have been done to date, the viability of *in vitro* produced embryos following transfer to the foster mother, is lower than *in vivo* produced counterparts. Among all variable that could influence the pregnancy outcome following transfer of *in vitro* produced sheep embryos, there is sparse evidence regarding to the effect of embryonic stage on embryo development and pregnancy outcome after embryo transfer. The aim of this study was to compare the pregnancy rates after transfer of *in vitro* produced lamb derived embryos, in two embryonic stages (morula and blastocyst), to the synchronized sheep.

MATERIALS AND METHODS

Except where otherwise indicated, all chemicals were obtained from the Sigma (St. Louis, MO, USA).

Oocytes collection and *in vitro* maturation: Prepubertal sheep ovaries were collected during the non-breeding season (June to July; 2006) from a local slaughterhouse and transported to the laboratory in saline at 30 to 35°C

within 1 to 3 h after collection. Follicular oocytes, aspirated from antral follicles with a 2-6 mm diameter, covered by at least three layers of cumulus cells and an evenly granulated cytoplasm was selected for *in vitro* maturation.

Groups of 15 cumulus-oocyte complexes were then allocated to 50 µL culture drops in a 60 mm Petri dish (Falcon 1008; Becton and Dickinson, Lincoln Park, NJ) containing maturation medium overlaid with mineral oil. The oocytes were incubated under an atmosphere of 5% CO₂-95% air with 100% humidity at 38.6°C for 24 h. The Oocyte Culture Medium (OCM) consisted of bicarbonate-buffered TCM 199 with L-glutamine supplemented with 0.02 mg mL⁻¹ cysteamine, 0.1 IU mL⁻¹ rhFSH (Organon International, Oss, The Netherlands), 1 IU mL⁻¹ hCG, 1 µg mL⁻¹ E₂, 100 µL mL⁻¹ penicillin, 100 µg mL⁻¹ streptomycin, 10% FCS and 0.2 mM Na-Pyrovate.

In vitro fertilization: Following maturation, oocytes were partially stripped of surrounding cumulus cells using a fine bore pipette and 300 IU mL⁻¹ of hyaluronidase in H-TCM199. Fresh semen was collected from a Lori-Bakhtiari breed ram of proven fertility. For swim up, 80-100 µL of semen was kept under 1 mL of BSA-HSOF (BSA-Hepes Synthetic Oviduct Fluid) in 15 mL conical Falcon tube at 39°C for up to 45 min. The 700-800 µL of the top fluid was then added to 2-3 mL of BSA-HSOF, centrifuged twice at 200 x g for 3 min. The final pellet was resuspended with BSA-HSOF and then added directly to the fertilization medium. The fertilization medium was modified SOF, enriched with 20% heated inactivated estrous sheep serum. A maximum of 10 oocytes per drop were fertilized in 40 µL with 1×10⁶ sperm mL⁻¹ under an atmosphere of 5% CO₂, 5% O₂ and 90% N₂ for 22 h at 38.6°C.

In vitro culture: After IVF, presumptive zygotes were allocated to 20 µL culture drops (five to six embryos/drop) consisting of SOF supplemented with 2% (v/v) BME-essential amino acids, 1% (v/v) MEM-nonessential amino acids, 1 mM glutamine and 8 mg mL⁻¹ fatty acid free BSA. The incubation conditions were 7% O₂, 5%CO₂ and 88% N₂ at 39°C in humidified air. On the third and fifth day of culture (with day 0 defined as the day of fertilization) 10% charcoal stripped Fetal Bovine Serum (FBS) was added to the medium. The culture was continued until 7-8 days post-fertilization.

Synchronization of recipients: The oestrous cycles of recipients (n = 9) were synchronized using an intravaginal progesteragen releasing devise (CIDR; Controlled Internal

Drug Release, 0.3 g progesterone; Pharmacia and Upjohn, Auckland). To induce follicular activity, recipients received 500 IU equine chorionic gonadotropin (eCG; Intervet, Boxmeer, The Netherlands) at pessary withdrawal on day 12.

Transfer of embryos: Following 4 to 7 day in culture, the embryos (at morula and blastocyst stage, respectively) were transferred surgically to the uterine horn of synchronized recipient ewes 4 and 7 days after the onset of oestrus, respectively.

A total of 9 ewes, because of the limited number of available animals, received 27 embryos (3 embryos/ewe). Of those, five ewes received 15 embryos at morula stage and four ewes received 12 embryos at blastocyst stage. Pregnancy was diagnosed by hormonal assay at 30 days and confirmed by ultrasonography at days 55 and 140. In order to investigate the full developmental potential of those embryos, pregnancies were allowed to go to term.

Statistical analysis: Comparison between groups was performed by the Fisher Exact test (SigmaStat, Jandel Scientific, SanRafael, CA, USA).

RESULTS AND DISCUSSION

The cleavage and blastocyst rates were 70 and 30.5% on the second and sixth day of culture (day 0 defined as the day of fertilization). As shown, among recipient ewes those received morula stage embryos just one was pregnant on day 30 (20%) which could not establish pregnancy after day 55. While from those received blastocyst stage embryos, three were pregnant on day 30 (75%) and two (50%) remained pregnant on each of days 55 and 148 (Table 1).

Following caesarian section (on day 148) two recipients delivered two lambs (4.6 and 3.9 kg).

In vitro- produced embryos have a lower survival rate (40%) and most embryo/fetal loss occurs at 30-35 days after transfer (Thompson, 1997). In this context, the pregnancy rate following transfer of fresh sheep IVP blastocysts was lower than that of *in vivo* embryos (54.3% versus 90.0%, respectively; Papadopoulos *et al.*, 2002). The incidence of early

Table 1: Pregnancy rate following transfer of lamb derived embryos produced *in vitro* in two embryonic stages

Embryonic stage	No. of pregnant recipients*				
	No. of embryos	No. of recipients	At 30 days (%)	At 55 days (%)	Term (%)
Compact morula	15	5	1(20)	0	0
Blastocyst	12	4	3(75)	2(50)	2(50)

*Day 0 defined as the day of fertilization

embryonic loss in adult sheep after natural mating is generally 20-30% and occurs predominantly during the first 5 weeks of pregnancy (Edey, 1976). In the current study, the higher percentage of embryonic and fetal loss during pregnancy, compared to some reports, could be related to the incompetence of lamb-derived embryos, especially at morula stage. Such pregnancy losses are a frequent feature following the transfer of juvenile-derived embryos in both sheep and cows (Revel *et al.*, 1995; Tervit *et al.*, 1995). In one study, the number of recipient ewes carrying embryos derived from 4-5 week-old lambs to term was around 20%, in contrast to reports of 38% pregnancy achieved with transferred embryos derived from oocytes from 10-12 week-old animals (Earl *et al.*, 1996). In several studies dealing with juvenile sheep embryo production, however, the pregnancy rate does not give a complete picture of the developmental capability of lamb oocytes (Earl *et al.*, 1995; Ledda *et al.*, 1997). One other possibility for higher pregnancy loss in the present study could be attributed to the adverse effects of heat stress on early embryonic development. Several studies have demonstrated heat to be a significant cause for reproductive problems in a wide variety of animals. These problems range from embryonic death and abortion to teratogenically induced anomalies and are heavily dependent on the dose and timing of the exposure (Edwards *et al.*, 1997; Graham and Edwards, 1998). Accordingly, in the current study, it seems the higher temperature and longer exposure during the summer months, at the time of pregnancies establishment, were the major causes for higher embryonic loss especially in those received morula stage embryos. Whether the morula stage embryos are more sensitive to heat stress than blastocyst stage embryos remain to be investigated. In the present study, the pregnancy rate on Day 30 following transfer of morula stage embryos was lower than transfer of blastocyst stage embryos (20 vs 75%). These percentages were decreased as pregnancy progressed and reached to 0 and 75% on day 55 and 0 and 50% on day 140, for morula and blastocyst stage embryos, respectively. The higher pregnancy rate following transfer of embryos in higher stage of development (blastocyst) could be explained by this hypothesis that they had been prospered from higher capability compared to the embryos in the lower stage of development (e.g., morula). There are, however, unsettled concerns about the safety of long term *in vitro* culture. In the current study the higher pregnancy rate after transfer of blastocyst than morula stage embryos is comparable with the results in some species. In human, blastocyst-stage transfer resulted in a significantly higher ongoing pregnancy rate (51.3 versus 27.4%) and live birth rate (47.5 versus 27.4%)

compared with day-3 embryo (morula stage) transfer (Papanikolaou *et al.*, 2005). A randomized study of embryo transfer after 3 (morula stage) or 5 days of embryo culture (blastocyst stage), however, resulted in 26% (60/233) and 25% (102/410) pregnancy rate, respectively. The average implantation rate per embryo was 13 and 12%, respectively (Scholtes and Zeilmaker, 1996). In buffalo the average pregnancy rate between morula and blastocyst (29.4 vs. 30.5%) was not very different (Campanile *et al.*, 1995). Whether the lamb-derived embryos in different embryonic stage exhibit different response to environmental stress such as heat stress and if there is difference in their compatibility to uterine environment after transfer, remained to be further investigated in this species. In conclusion the viability of *in vitro* produced ovine embryos following transfer is increased as the embryos develop to the blastocyst stage. Although allowing embryos to develop to the blastocyst stage may confer this advantage and higher survival rate, there are still unsettled concerns about the safety of long term exposure to the media and other factors in *in vitro* culture.

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