Preliminary report on pregnancy rates after transfer of cryopreserved and fresh embryos produced in vivo and in vitro in sheep

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ABSTRACT

Continuous improvements to assisted reproductive technology (ART) have facilitated the advancement and preservation of animal genetics as well as animal reproductive efficiency. One of the ART techniques is cryopreservation which facilitates embryo transfer procedures. In the present experiment embryos produced in vivo or in vitro were transferred to the recipient ewes just after collection (fresh) or after freezing/thawing on day 5 after fertilization. Pregnancy was verified 40 or more days after embryo transfer (ET) by ultrasonography. For in vivo produced embryos, transfer of fresh embryos resulted in a 43% (3 out of 7) pregnancy rate, but transfer of frozen/thawed embryos resulted in 11% (1 out of 9) pregnancy rate. For in vitro produced embryos, transfer of fresh embryos resulted in a 50% (2 out of 4) pregnancy rate, but transfer of frozen/thawed embryos resulted in no pregnancies (0 out of 6). These data demonstrate that the cryopreservation method and ET procedures need additional improvements, since pregnancy rates remained at a low level.

INTRODUCTION

The first successful report on the freezing of mammalian embryos was that of Whittingham in 1971. A few years later in 1976, the first birth from a frozen sheep embryo was reported (Willadsen et al., 1976). The sheep has been used as a model for studies on freezing the embryos of other domestic species, especially cattle (Willadsen et al., 1976, 1977; Bilton and Moore, 1976). Since 1995, numerous studies have reported production of live offspring following cryopreservation of sheep embryos (Songasasen et al., 1995; Cocero et al., 1996; Loi et.al. 1998).

Cryopreservation of embryos plays a key role in commercial embryo technology and has become an integral part of methods to control animal reproduction. The growth of clinical services for IVF has been a major stimulus for cryotechnology (Leibo et al., 1989).

This technique brings numerous advantages. As an example, it allows for the long-term storage of embryos when the number of recipients is limited or when the production of live offspring needs to be postponed to a later date (Martinez at al., 1997; Fahning et al., 1992). Also, embryo freezing offers the potential for full development of embryos that would otherwise be discarded. In this way, using in vitro production of embryos enables the production of a large number of offspring from one animal. Furthermore, this method can preserve genetic material from valuable animals and can help in the management of infertility. In addition, frozen embryos can be easily transported from one place to another (Leibo, 1989; Ali et al., 1993; Martinez e al., 1997).

Although embryo cryopreservation has been successful, many problems remain. Notable among these is the low survival of embryos after freezing. Despite subsequent progress, freezing and thawing is not completely reversible because of lost viability, and storage of the embryos still remains a difficulty. The advantages of embryo cryopreservation must be weighed against its known and potential disadvantages (Kutluk et al., 1998).

The aim of this study was to compare the pregnancy rates after transfer of cryopreserved and fresh embryos produced in vivo and in vitro in sheep.

MATERIALS AND METHODS

Animals

White-faced, predominantly Targhee and Rambouillet crossbred ewes that exhibited an estrous cycle of normal duration (15-17 days) were used for this study during the normal reproductive season (October-December, 2002 and 2003). Ewes were checked for estrus twice daily by using vasectomized rams. Protocols for procedures used in this study and animal care were approved by the Institutional Animal Care and Use Committee.

Donors. For collection of in vivo produced embryos, ewes (n = 10) were injected twice daily (morning and evening) with FSH-P (Sioux Biochemical, Sioux Center, IA; Jablonka-Shariff et al., 1994; Stenbak et al., 2001; Grazul-Bilska et al., 2003) on days 13, 14 and 15 of the estrous cycle, and then bred naturally with a Hampshire ram of proven fertility. For collection of in vitro produced embryos, ewes (n = 6) were injected with FSH on days 13 and 14 of the estrous cycle. On the morning of day 15, ovariectomy was performed to collect ovaries (Reynolds et al., 1998).

Recipients. Ewes (n=27) were randomly selected to receive embryos on day 5 of the estrous cycle. The estrous cycles of the recipient ewes were synchronized so that their expected day of ovulation coincided with the day of breeding or in vitro fertilization (IVF). The synchronization method consisted of two (morning and evening of the same day) i.m. injections of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$; Estrumate, Schering-Plough Animal Health Corp., Union, NJ., 125 mg/ml/injection) on day 8-12 of the estrous cycle. Estrus was detected 36-48 h after the second $PGF_2\alpha$ injection. For in vivo produced embryos, fresh embryos were transferred to 7 recipients, and frozen/thawed embryos to 9 recipients. For in vitro produced embryos, fresh embryos were transferred to 4 recipients, and frozen/thawed embryos to 6 recipients.

Embryo and oocyte collection and in vitro fertilization procedures.

In vivo produced embryos were collected by uterine flushing (Ptak et al., 1999; Dattena et al., 1999). Ovaries from donor animals were collected and fertilized in vitro as previously described by Grazul-Bilska et al. (2003). Following ovariectomy, ovaries were transported to the laboratory and oocytes were isolated by opening each visible follicle with a scalpel blade and flushing it two to three times with oocyte collection medium (Stenbak et al, 2001; Grazul-Bilska et al., 2003). Under a stereomicroscope, oocytes were recovered from each dish and transferred to a petri dish containing fresh collection medium without heparin. Oocytes were then evaluated and categorized as healthy or atretic based on morphology (Thompson et al., 1995). All oocytes were washed three times in maturation medium (TCM-199 containing 10 % FBS, ovine FSH [oFSH-RP-1; NIAMDD-NIH, Bethesda, MD], ovine LH [oLH-26; NIADDK-NIH], estradiol [Sigma St. Louis, MO], glutamine [Sigma], sodium pyruvate [Sigma], EGF (10 ng/ml [Sigma]) and penicillin/streptomycin; Stenbak et al., 2001; Grazul-Bilska et al., 2003).

Oocytes were matured for 24 h at 39ºC in 5% CO_2 and 95% air followed by cumulus cells removal by using a 1% hyaluronidase (Type I-S; Sigma) treatment. The oocytes were again evaluated for health based on morphology (Stenbak et al, 2001; Grazul-Bilska et al., 2003). Oocytes classified as healthy were used for IVF and were transferred to stabilized fertilization

medium consisting of synthetic oviductal fluid (SOF) and 2% heat inactivated sheep serum collected from sheep on day 0-1 of the estrous cycle (Grazul-Bilska et al., 2003).

Frozen capacitated semen pooled from 4 Hampshire rams, was thawed and viable sperm were separated using the swim up technique (Yovich 1995; Stenbak et al., 2001). The sperm ($0.5\text{-}1.0x10^6$ sperm/ml) were added to the IVF medium containing oocytes and incubated for 18 h at 39ºC, 5% O_2, 5% CO_2 and 90% N_2. The oocytes/zygotes were then washed three times with culture medium without glucose (SOF supplemented with BSA, glutamine, MEM amino acids, BME amino acids [Sigma] and penicillin/ streptomycin; (Grazul-Bilska et al., 2003), and cultured in the same medium for 24 h at 39ºC, 5% O_2, 5% CO_2 and 90% N_2. The dishes were then evaluated to determine the number of fertilized oocytes. The embryos were transferred to culture medium containing glucose (Grazul-Bilska et al., 2003). After 48 hours, the developmental stage of the zygotes was evaluated and embryos were transferred to the fresh culture medium with glucose. On day 5 of culture (day 1 = day of fertilization) embryos in the stage of 16 or more cells were randomly selected for transfer.

Embryo freezing (cryopreservation)

Embryos (16 cells to early morula stage) selected for embryo transfer were collected in holding media (Agtech; Manchattan, KS), then transferred to dishes containing 75% of holding media and 25% of freezing media (Agtech) for 5 minutes followed by transfer to a dish containing 50% of each media for 5 minutes and next to dish with 25% holding media and 75% freezing media for 5 minutes and then to a dish with freezing media only. 2-3 embryos in freezing media were loaded per each straw. After 10 minutes, a slow freezing procedure was performed using a freeze control CL-8000 system (CryoGenesis, Australia).

Embryo transfer

Recipient ewes were anesthetized by an i.m. administration of 0.4 ml Rompun (20 mg/ml dose) and 2.0 ml of ketamine hydrochloride (100 mg/ml Ketaset). Ewes were restrained on their backs and the abdominal cavity was inflated with CO_2. A 1.5 mm diameter laparoscope was inserted through a 2 mm trocar approximately 5 cm anterior to the udder, and approximately 2 cm lateral to the midline. The ovary containing the functional corpora lutea (CL) was identified.1.5-2 cm incision was then made through the skin and body wall adjacent to the laparoscope and approximately 2-5 cm lateral to the midline. A Babcock forceps was inserted through this incision and the anterior portion (approximately 2-4 cm) of uterine horn ipsilateral to the ovary with the CL was grasped and exteriorized. A Sovereign® Tom Cat catheter (Sherwood Medical, St Louis, MO, USA) containing two or three embryos in approximately 3 µl of culture medium was inserted through a small incision and the embryos were expelled to the uterine lumen. The uterine horn was then rinsed with sterile saline and replaced into the abdomen. The abdominal incision was then closed with 1-2 sutures.

Pregnancy determination

The recipient ewes were placed with vasectomized rams beginning on day 6 after ET to check for estrus. Ewes exhibiting estrus activity were determined to be non-pregnant. In addition,

the presence of fetuses was determined for all recipients using ultrasonography (Classic Ultrasound Equipment Ltd, Tequesta, FL) on day 40 or later after ET.

RESULTS

Pregnancy rates after transfer of in vivo or in vitro produced embryos were similar. Pregnancy rates after transfer of frozen-thawed embryos were lower than those achieved after transfer of fresh embryos produced in vivo or in vitro. Transfer of frozen/thawed embryos produced in vitro resulted in no pregnancies (Table 1).

Table 1. The pregnancy rates after transfer of cryopreserved or fresh embryos produced in vivo and in vitro

	Fresh embryos			Frozen embryos		
	Recipients (n)	Pregnant sheep (n)	Pregnancy rate (%)	Recipients (n)	Pregnant sheep (n)	Pregnancy rate (%)
In vivo	7	3	43%	9	1	11%
In vitro	4	2	50%	6	0	no pregnancies

DISCUSSION

Results of the present study demonstrated that embryos produced in vitro are more sensitive to cryopreservation and have significantly reduced pregnancy rates (0%) after embryo transfer than in vivo produced sheep embryos (11%). However, transfer of fresh embryos produced in vivo or in vitro resulted in similar pregnancy rates which suggest that in vitro conditions provided were similar to in vivo conditions for early embryonic development.

Embryo transfer procedures in sheep have been successfully used by numerous researchers (Ishwar and Memon, 1996). Pregnancy rates after fresh embryo transfer reported by others varied from 29 to 69% in sheep (Rexroad and Powell; 1990; Slavik et al., 1992; Thopmson et al., 1995; Brown et al., 1998; Ptak et al., 1990; Dattena et al., 2000). In our laboratory, the rates of pregnancy after embryo transfer remain at the range of 43-52% (presented study, Pant et al., 2003)

There preliminary results indicate that futher studies are needed to improve embryo cryopreservation. It has been suggested that in vitro produced (IVP) embryo survival after cryopreservation, both in vivo and in vitro may be a good evaluation of the true competence of IVP embryo production systems (Dobrinsky et al., 2002). Although cryopreservation can enhance the utilization of in vitro embryo production technologies, cryosurvival of in vitro-produced (IVP) embryos is less than that of in vivo-derived embryos.

In this study, we were using embryos at 16-cells to early morula stage for embryo transfer. Although the rates of pregnancy after ET of fresh embryos were acceptable, pregnancy rates after frozen/thawed embryos were very low. The effect of the stage of embryo development can be a principal factor affecting successful cryopreservation of mammalian embryos (Cseh et al., 1997). Cseh et al. (1997) reported significant differences in the survival and development of mouse embryos cryopreserved at different developmental stages including 2 cell, 4 to 8-cell, compacted

morula, early blastocyst, expanding blastocyst and expanded blastocyst stages. Their results showed the highest survival (80%) for morula and early blastocyst stage. It was also previously demonstrated that pregnancies rates can be increased by choosing embryos in the blastocyst stage after additional selection based on embryo viability before transfer (Cognie et al., 1999). Successful cryopreservation of blastocysts utilizes only the highest ranked blastocyst for freezing and thawing (Kuwayama et al., 1992). This indicates, that the low pregnancy rates obtained in our study were likely caused by the stage of embryo development.

Previous experiments done by others show the influence of the type of cryoprotectant, concentration and also temperature on embryo survival (Fuku et al., 1992; Lim, et al., 1999). From their results it is clear that early blastocysts showed substantially greater survivability than morulas and in general, survival of embryos of either stage increased with the concentration of cryoprotectant, while the proportion of embryos surviving decreased with decreasing storage temperature and with increased duration of storage. In our study, we have used holding and freezing media commercially prepared and freezing procedures successfully used by others (Gordon, 1997).

Further studies should consider improvement of embryo survival after freezing which can be achieved by changing the condition of culture, selection of embryos based on the stage of their development, and changing cryopreservation procedures (Rizos et al., 2001; Massip et al., 1995). Survival rates of in vivo produced embryos can be significantly improved by a controlled rate cooling and warming (Hasler et al., 1997; Hochi et al., 1996; Jackson et al., 1997). In vitro produced embryos were shown to be more sensitive to decrease temperatures than in vivo counterparts (Han et al., 1994; Leibo et al., 1993).

In conclusion, our preliminary results demonstrate that low pregnancy rates following transfer of cryopreserved embryos are probably being affected by using embryos too early in development (i.e., 16-cells to early morula stage). This strongly suggested that the stage of embryo development as well as embryo production condition play a crucial role in the survival of embryos after cryopreservation. Although the number of embryos used in this studies were low, these data nonetheless indicate the importance of more research in this area, and provides valuable insight into the next step for obtaining increased efficiency of described procedures.

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