İstanbul Üniv. Vet. Fak. Derg.
36 (1), 1-8, 2010
Araştırma Makalesi

J. Fac. Vet. Med. İstanbul Üniv.
36 (1), 1-8, 2010
Research Article

Production of Cloned Lambs: Transfer of Early Cleavage Stage Embryos to Final Recipients

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Geliş Tarihi /Received: 10.12.2009

ABSTRACT

The aim of this study was to obtain cloned lambs by somatic cell nuclear transfer. In vitro matured oocytes were enucleated and injected with serum-starved cumulus cells. After electrofusion and ionomycin treatments, reconstructed oocytes were activated by 10 µg/ml cycloheximide (CHX) for 5 h or 2 mM 6-dimethylaminopurine (6-DMAP) for 3 h and cultured in vitro in SOF medium at 38.5°C in a humidified atmosphere of 5% CO_2, 5% O_2 and 90% N_2.

Cleavage rates were 18.1% (26/144) in CHX and 46.5% (140/301) in 6-DMAP groups; 11.4% of embryos developed to the morula stage in the 6-DMAP group. To investigate the developmental capacity of the embryos to become live young, early cleavage stage embryos (n= 69) were transferred to the oviducts of synchronized recipients. Five out of eight ewes (62.5%) were diagnosed as pregnant at Day 18 according to progesterone assays, and two ewes (25.0%) were diagnosed as pregnant after 45 days according to ultrasound examinations. Two healthy lambs were born by Cesarean section.

In conclusion, although by using different activation protocols blastocyst stage embryos were not obtained after in vitro culture of the somatic cell nuclear transfer embryos, live young that were born after transfer of early stage embryos showed that these embryos possess developmental capacity.

Key Words: sheep, somatic cell nuclear transfer, early transfer

Introduction

Twelve years after the birth of Dolly, the first cloned lamb, somatic cell nuclear transfer (SCNT) is still of tremendous interest to researchers. The very low success rate of this procedure, however, restricts its use for broad purposes. Although SCNT was first announced in sheep, SCNT experiments in sheep have been rare. The problems encountered with sheep SCNT are low success rates with in vitro matured oocytes (Campbell et al., 2007), low rates of blastocyst formation after in vitro culture of reconstructed embryos (Loi et al., 1999) and high mortality rates (Ptak et al., 2002). The developmental rate of reconstructed embryos is affected by many conditions, such as oocyte and donor cell sources, electrofusion and activation treatments and culture conditions. Although different MPF inhibitors are used for oocyte activation, developmental failures have been reported for all inhibitors (reviewed in Alberio et al., 2001). Alexander et al. (2006) showed no differences in blastocyst frequencies or chromosomal abnormalities of the SCNT embryos that were activated by CHX versus 6-DMAP. Rather than in vitro culture, in vivo culture of reconstructed oocytes is generally used to increase rates of development to the blastocyst stage in sheep SCNT embryos (Loi et al., 1998; 1999; Ptak et al., 2002). Lost embryos after recovery (Loi et al., 1998; 1999) and the need for surgery are the main problems associated with in vivo culture.

In this study, two different activation protocols were used for obtaining sheep SCNT embryos, and early stage embryos were transferred into final recipients to produce cloned lambs.

Materials and methods

All chemicals were purchased from Sigma unless otherwise specified. All animal treatments were in compliance with animal experimental ethics at the University of Istanbul.

Donor cell preparation and somatic cell nuclear transfer

Five or six cumulus-oocyte complexes (COCs) that were obtained from an ovary of a slaughtered Kıvırcık ewe were gently pipetted, and cumulus cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 mM L-glutamine, 1 mM Na-pyruvate, 1% (v:v) MEM non-essential a.a., 75 µg/ml penicillin G, 50 µg/ml streptomycin and 15% FCS (v:v). After 1-3 passages, serum-starved (0.05% FCS) cells of at least 4 days were used as karyoplasts (Peura and Vajda, 2003).

COCs were obtained and matured as reported previously (Birler et al., 2002a; 2002b). Briefly, COCs were recovered from sheep ovaries of slaughtered ewes and matured for 18-20 h in medium 199 supplemented with 10% FCS, 0.1 mg/ml Na-pyruvate, 10 µg/ml of FSH and 10 µg/ml LH at 38.5°C in a humidified atmosphere of 5% CO_2.

After denudation by vortexing in synthetic oviduct fluid (SOF) medium with HEPES (HSOF) plus 600 IU/ml hyaluronidase for 3-5 min, M II oocytes were selected. Before enucleation, oocytes were stained with Hoechst 33342 (7.5 µg/ml in HSOF) and exposed briefly to fluorescent light to locate the metaphase plate. Somatic cell-oocyte couplets were exposed to 1.4 kV/cm for 40 µsec, 2 pulses (BTX 830) in mannitol-based fusion medium (Alexander et al., 2006). After incubation in 5 µM ionomycin for 5 min, reconstructed oocytes were left in SOF medium supplemented with 2 mM 6-DMAP for 3 h or 10 µg/ml CHX for 5 h at 38.5°C in a humidified atmosphere of 5% CO_2, 5% O_2 and 90% N_2. Embryos were cultured in SOF medium for 8 days. On Day 3, 1.5 mM glucose was added to the culture media droplets.

Embryo transfer, pregnancy control and birth of cloned lambs

Healthy mature Kıvırcık ewes were synchronized using intravaginal sponges impregnated with 20 mg flugestone acetate (Chronogest CR; Intervet, France) for 14 days; 400-500 IU PMSG (Chronogest; Intervet, Netherlands) were given i.m. at the time of sponge withdrawal. Forty-eight hours after sponge withdrawal was designated as Day 0. All cleaved SCNT embryos at Day 2 were transferred into the oviducts ipsilateral to the corpus luteum-bearing ovary of recipient ewes by midline laparotomy. Operations were performed under xylazine sedation and lumbosacral anesthesia. Blood samples were taken at Day 18 of pregnancy. Recipients with serum progesterone levels above 1 ng/ml were considered to be pregnant. Pregnancies were confirmed by ultrasound examinations after 45 days.

Pregnant ewes were given 2 mg estradiol benzoate followed by 4 doses of betamethasone disodium phosphate, 12 h apart, at Day 146 of pregnancy. Cesarean operations were performed on Day 148. Live young were given 1.5 mg dexamethasone immediately after birth (Ptak et al., 2002).

Microsatellite analyses

The DNA genotypes of the cloned lambs, recipients and the donor sheep's ovarium were tested with the following 9 microsatellite loci: JMP29, JMP58, ILSTS005, OarFCB20, OarFCB48, MAF65, MAF209, MCM140 and DYMS1. Blood samples of cloned lambs and recipients were collected into 9 ml K_3EDTA containing vacuum tubes. DNA was isolated using a standard phenol: chloroform extraction method (Sambrook et al., 1989). DNA of the donor sheep was isolated from a small piece of ovary; the protocol provided by Nagaya University's Medaka Book (Takeda, 2007) and the protocol by Taggart et al. (1992) were modified and employed. For the microsatellite-based genotyping, each forward primer was labeled at its 5' end with one of three dye labels: FAM, TET or HEX. Electropherograms were analyzed using Genotyper software.

Statistical analyses

Statistical analysis was performed using a Chi-square test by SPSS for Windows version 13.0.

Results

Reconstructed oocytes (n= 445) were randomly divided into two activation groups: CHX (n= 144) and 6-DMAP (n= 301). Although the embryos in the 6-DMAP group developed to the morula stage, the embryos in the CHX group did not. Results obtained for the effects of activation treatment are given in Table 1.

Table 1. Effects of activation treatment on the development of SCNT sheep embryos.

Activation Treatment	N	Cleavage (%)	Morula (%)	Blastocyst (%)
CHX	144	26 (18.1)[b]	–	–
6-DMAP	301	140 (46.5)[a]	16 (11.4)	–

a, b: Values with different superscript within column differ significantly ($P<0.001$).

For SCNT embryo transfer, all cleaved embryos (n= 69) were transferred into 8 recipient ewes (6-11 embryos per recipient). Embryo transfer and pregnancy results are given in Table 2. Five out of eight ewes (62.5%) were diagnosed as pregnant at Day 18 according to progesterone assays, and two ewes (25.0%) were diagnosed as pregnant after 45 days according to ultrasound examinations; one of them had four fetuses.

Table 2. The results of early transfer of SCNT embryos to final recipient ewes.

Rep. No	Rec. oocytes	Cleaved & Transferred (%)	Recipient No	Pregnant Ewes (%)		Fetuses (%)*	Live Young	
				At D 18	After D 45		Born (%)*	Survive**
3	173	69 (39.9)	8	5 (62.5)	2 (25.0)	5 (7.2)	2 (2.9)	2

Rep.: replication; Rec.: reconstructed; D: day; *: from early cleavage stage embryos; **:after 1 yr.

Two pregnancies continued to term and 2 (2.9%; according to cleavage stage embryos) healthy cloned lambs (named Oyalı and Zarife; Figure 1) were born by Cesarean section at D 148 of pregnancy. Birth weights of cloned lambs were 5.6 and 3.7 kilograms, respectively (Table 3). One mummified (Figure 2) and one macerated fetuses were also delivered from Zarife's surrogate mother. DNA of the cloned lambs Oyalı and Zarife, their recipients and the donor sheep's ovary were genotyped using 9 microsatellite loci; according to these results, Oyalı, Zarife and their donor had the same genotype (Table 4).

Figure 1. Oyalı and Zarife (after 1 year)

Table 3. Weight changes (kg) of cloned lambs (Oyalı and Zarife) from born to weaning.

	Birth weight (kg)	Weeks							
		1	2	3	4	5	6	7	8
Oyalı*	5.60	7.20	9.75	11.74	14.50	17.75	21.18	21.65	25.15
Zarife**	3.73	6.00	7.89	10.87	13.70	16.54	19.20	21.90	25.80

*: Birth date (21.11.07); **: Birth date (28.11.07)

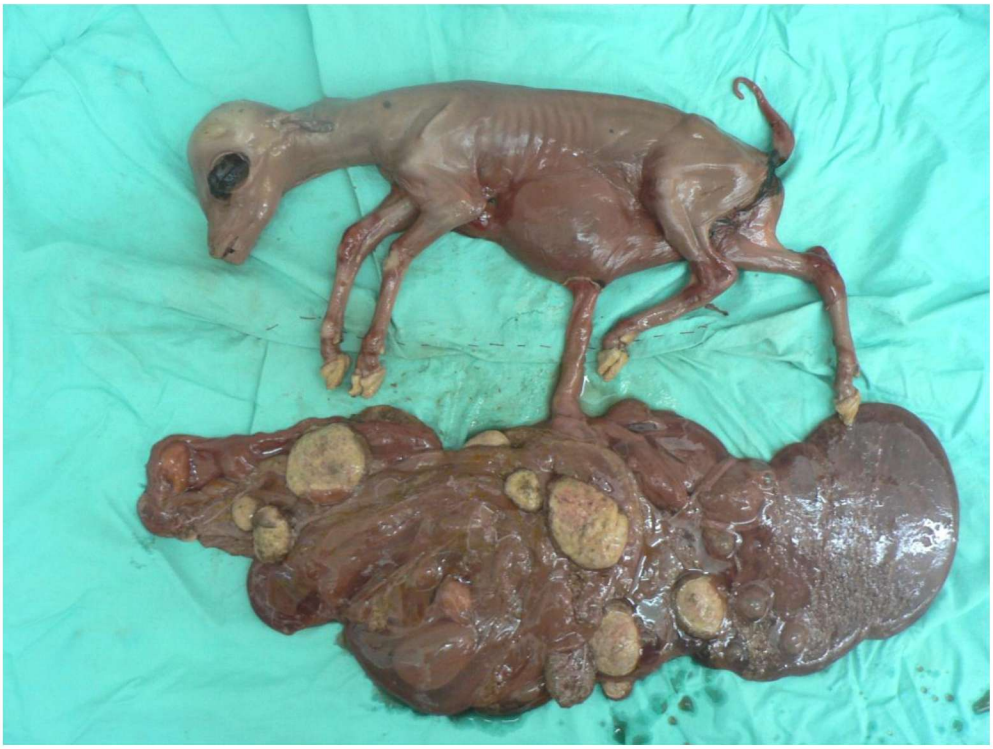

Figure 2. Mummified fetus.

Table 4. Microsatellite analysis results of samples. Numbers indicate the alleles (in terms of the sizes of the observed fragments in base pairs) of the locus.

Microsatellite Loci	Donor ovary	Oyalı	Zarife	Zarife's Recipient	Oyalı's Recipient
DYMS1	181-195	181-195	181-195	181-181	181-181
ILSTST005	192-199	192-199	192-199	187-199	197-199
JMP29	131-131	131-131	131-131	124-129	133-133
JMP58	138-147	138-147	138-147	140-150	140-165
FCB20	97-106	97-106	97-106	95-101	108-114
FCB48	147-147	147-147	147-147	149-165	149-165
MAF65	125-135	125-135	125-135	129-135	127-131
MAF209	114-114	114-114	114-114	112-114	112-114
MCM140	184-186	184-186	184-186	180-184	184-186

Discussion

The success rate of somatic cell nuclear transfer in sheep is strongly influenced by factors such as oocyte source (Campbell et al., 2007; Peura and Vajda, 2003; Wells et al., 1997) and culture conditions (Alexander et al., 2006; Campbell et al., 2007; Peura and Vajda, 2003). While some researchers have obtained high blastocyst rates using in vitro culture for culturing SCNT embryos (15-25%; Alexander et al., 2006; Wells et al., 1997), some have preferred in vivo culture in sheep oviducts to obtain higher rates (Loi et al., 1998; 1999). Related to the low success rate with slaughterhouse oocytes, in vivo matured oocytes are generally preferred by researchers

(Loi et al., 1998; Ptak et al., 2002; Wells et al., 1997).

In this study, blastocyst-stage embryos were not obtained after in vitro culture of SCNT embryos. Low cleavage rates obtained in NT group may be related to the cytoplast sources.

Although the SCNT embryos produced in this study did not develop to the blastocyst stage after in vitro culture, to our knowledge, this is the first study in which live young have been obtained by the transfer of sheep SCNT embryos to final recipients immediately after the first cleavage divisions. Keefer et al. (2001) concluded that early transfer of caprine embryos results in very high rates of pregnancy and live young; this phenomenon was thought to be species-specific. Similar results obtained by early transfer of ovine embryos in this study reveals that positive results after early transfer of cloned embryos are not species-specific. In vitro maturation and in vitro culture of embryos are the limiting factors for SCNT and also IVP in sheep (Birler et al., 2002a; Walmsley et al., 2004). This is consistent with the hypothesis that placing early stage embryos into oviducts may protect the embryos from unpredictable and undesirable culture conditions that may perturb further development (Khosla et al., 2001; King et al., 2002; Mahsoudi et al., 2007; Tveden-Nyborg et al., 2005; Walker et al., 1996).

Recognition of embryos by their mothers is a key process for future destination of pregnancies. Embryo implantation process is not fully understood, but it is believed that oviduct has a very important role in this process secreting important proteins selectively (Georgiou et al., 2007; Huang et al., 2004; Sostaric et al., 2006).

Birth weights of the cloned lambs, Oyalı and Zarife were 5.60 and 3.73 kilograms respectively. Whereas Oyalı's birth weight was greater than breed characteristics, she had normal development after birth without any health problem (Table 3). Greater birth weights obtained in IVP lambs in our previous study (Corekci et al., 2004) did also not influence further development of lambs.

Pregnancy and live young rates obtained in the present study confirmed that early stage transfer of SCNT embryos will not regarded as impracticable considering high costs of recipients, complications and being less repeatable (Campbell et al., 2007; Walmsley et al., 2004). Furthermore comparable pregnancy and high live young rates were obtained indicating that embryos could develop more successfully to the implantation stage in vivo. Early transfer could lead lower cost and welfare problems than in vivo or in vitro culture of cleaved embryos up to the blastocyst stage, as well.

In conclusion, the results of this study reveal that activation treatment by 6-DMAP gives better results for sheep oocytes and that sheep SCNT embryos can develop into live young if they are transferred after the first cleavage divisions. Because in vivo or in vitro culture of reconstructed embryos up to the blastocyst stage require a great deal of time, money and effort, and development of the embryos is better for in vivo than for in vitro conditions, early stage transfer of sheep reconstructed embryos may be used.

Acknowledgements

This project was supported by the State Planning Organization (SPO; Project No: 2003K120680), the Scientific and Technical Research Council of Turkey (TUBITAK; Project No: 105 O 709) and the Research Fund of Istanbul University (Project No: UDP-1643). The authors would like to thank Dr. K. F. Ertekin, Prof. S. Meinecke-Tillmann, Prof. M. C. Gomez and Dr. G. Ptak for their invaluable comments about induction of parturition of the recipients. The authors would also like to give special thanks to Assoc. Prof. H. Bağış and S. Arat for their comments in preliminary studies. Many thanks go to Prof. R. Kahraman, Prof. A. Kaymaz, B. Dokuzeylül, L. Koenhemsen, O. İskefli, D. Aydın, D. J. Şadalak, F. Esen, V. Kayar, G. Kaya, A. K. Gürbüz and A. Günay

for their help with the care and management of the recipients and cloned lambs.

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