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Interspecies Nuclear Transfer of Tibetan Antelope Using Caprine Oocyte as Recipient

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ABSTRACT Interspecies nuclear transfer is an invalulable tool for studying nucleus–cytoplasm interactions; and at the same time, it provides a possible alternative to clone endangered animals whose oocytes are difficult to obtain. In the present study, we investigated the possibility of cloning Tibetan antelope embryos using abattoir-derived caprine oocytes as recipients. Effects of culture conditions, enucleation timing, and donor cell passages on the in vitro development of Tibetan antelope-goat cloned embryos were studied. Maternal to zygotic transition timing of interspecies Tibetan antelope embryos was also investigated using two types of cloned embryos, Tibetan antelope-rabbit and Tibetan antelope-goat embryos. Our results indicate that: (1) goat oocyte is able to reprogram somatic cells of different genus and supports development to blastocyst in vitro. (2) Coculture system supported the development of Tibetan antelope-goat embryos to blastocyst rate stage (4.0%), while CR1aa alone did not. (3) When MII phase enucleated caprine cytoplast and TII phase enucleated caprine cytoplast were used as recipients, the fusion rate and blastocyst rate of hybrid embryos were not statistically different (73.9% vs. 67.4%; 4.0% vs. 1.1%). (4) When donor cells at 3–8 passages were used, 2.9% hybrid embryos developed to blastocysts, while none developed to blastocysts when cells at 10–17 passages were used. (5) There may be a morula-to-blastocyst block for Tibetan antelope-goat, while there may be an 8- to 16-cell block for Tibetan antelope-rabbit embryos. *Mol. Reprod. Dev. 74: 412–419, 2007.* © 2006 Wiley-Liss, Inc.

Key Words: Tibetan antelope; interspecies cloning; maternal to zygotic transition

INTRODUCTION

The Tibetan antelope (*Panthalops hodgsonil*), which is included in CITES list of endangered species, is mainly distributed in Tibet and Qinghai of China, and its survival is seriously threatened despite efforts to maintain the limited populations via habitat and wildlife conservation. Recent advances in assisted reproductive techniques, such as cryogenics of gametes/embryos, artificial insemination, and embryo transfer have allowed the further propagation of endangered species. Since the birth of Dolly (Wilmut et al., 1997), successful somatic cell nuclear transfer has been achieved in 11 species (Wilmut et al., 1997; Cibelli et al., 1998; Kato et al., 1998; Wakayama et al., 1998; Baguisi et al., 1999; Onishi et al., 2000; Polejaeva et al., 2000; Chesne et al., 2002; Shin et al., 2002; Galli et al., 2003; Woods et al., 2003; Zhou et al., 2003; Lee et al., 2005).

The technique of somatic cell nuclear transfer provides not only a valuable tool to multiply animals of the same genetic traits, but a prospective alternative to the preservation of endangered species. However, the traditional somatic cell nuclear transfer is inefficient in this regard at present. The lack of endangered species-specific oocytes and recipients make the scientists to find interspecies nuclear transfer alternative. The success of interspecies cloning of gaur and mouflon further have demonstrated the feasibility to preserve endangered species or restore extinct species using interspecies nuclear transfer (Lanza et al., 2000; Loi et al., 2001). Previous studies have shown that oocyte cytoplasm from bovine, sheep, and rabbit is able to dedifferentiate somatic cell from different species and supports early development of these interspecies nuclear transferred embryos to blastocyst (Dominko et al., 1999; White et al., 1999; Chen et al., 2002).

At present, Tibetan antelope produced by intra-or interspecies somatic cell nuclear transfer has not been reported. It is not clear whether or not the Tibetan antelope somatic cell could dedifferentiate in enucleated goat oocyte. In the present study, we explore the

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possibility of Tibetan antelope embryo cloning using abattoir-derived goat oocytes as recipients and investigate the in vitro and in vivo development potential of cloned Tibetan antelope embryos.

MATERIALS AND METHODS

Chemicals

All the chemicals used in the experiments were purchased from Sigma Company (St. Louis, MO) except for those specifically noted.

Culture of Donor Cells

Cell culture and assessment procedure have been described previously (Han et al., 2001). Briefly, ear skin sample was obtained by biopsy from a 10-month-old female Tibetan antelope. Tissues were manually cut into small pieces measuring about 1 mm^2 and digested with 0.25%(w/v) trypsin (Gibco BRL, Grand Island, NY) for 12 hr at 4°C and then for 30 min at 37°C, and then the digested cells and tissues were seeded into 75-cm^3 cell culture flask containing Dulbecco's modified Eagle's medium/F-12(DMEM/F-12;Gibco) supplemented with 20% fetal bovine serum (FBS; Gibco) and cultured in a 5% CO_2 incubator at 37°C. After reaching 75–85% confluence, monolayers of the primary cells were disaggregated for further culture. A proportion of the cells at passages 1–3 were frozen and stored in liquid nitrogen for long-term use. Fibroblast cells at passages 3–8 or 10–17 were used as donor cells.

Chromosome Analysis

To examine the ploidy of the cultured somatic cells at various passages, chromosome counts were determined at passages 3–8 and 10–17 using standard preparation of metaphase spreads. In brief, cells were treated with demecolcine (0.04 μg/ml) for 3 hr at 37°C in an atmosphere of 5% CO_2 and 95% air. After demecolcine treatment, the cells were trypsinized and centrifuged and the supernatant was removed. Cells were resuspended in a prewarmed hypotonic solution (0.075 mol/L KCl) and incubated at 37°C for 30 min. They were then centrifuged and the resulting pellet resuspended in 5 ml of fixative (3:1 methanol:glacial acetic acid) at room temperature (RT) for 15 min. Fixed cells were centrifuged and washed twice in fresh fixative. After a final centrifuge, the cells were resuspended in 0.1 ml fresh fixative and single drops were dropped on clean slides and air-dried. Slides were stained with 5% Giemsa for 10 min, rinsed with distilled water, and air-dried. The numbers of well spread chromosomes within a clear cell boundary were counted under a light microscope at 1,000× magnification under oil.

Oocyte Collection and In Vitro Maturation

Goat ovaries were collected from a local abattoir and transported within 3 hr to the laboratory in sterilized saline containing 100 IU/ml penicillin and 0.05 mg/ml streptomycin and maintained at 30–35°C. Ovaries were washed three times with warmed Dulbecco PBS (DPBS) and cumulus oocyte complexes (COCs) were released from the follicles by cutting the ovaries with sharp bistouries in DPBS plus 1% FBS and 10 μg/ml heparin. The COCs were examined under a stereomicroscope and only those with more than four layers of cumulus cells and a finely granulated homogeneous ooplasm were selected for in vitro maturation (IVM). Selected COCs were washed three times in M199 maturation medium and then were cultured in groups of around 20 in 100 μl droplets of TCM199 maturation medium supplemented with 10% FBS, 10 μg/ml FSH, 5 μg/ml LH, and 1 μg/ml 17-β estradiol for 24 hr at 38.5°C under 5% CO_2 in humidified air. Following IVM for 24 hr, oocytes were denuded of cumulus cells by incubation in 0.5% hyaluronidase and repeated gentle pipetting. Only oocytes with the first polar body, or partially extruding second polar body and a finely granulated ooplasm were selected for subsequent experiments. In some experiments, MII arrested caprine oocytes were used as recipients.

Mature female rabbits were superovulated by administering PMSG and hCG (Institute of Zoology Chinese Academy of Sciences). Each rabbit was injected with 150 IU PMSG and 100 IU hCG 96 hr after the PMSG injection. Rabbits were killed 14–15 hr after hCG injection. Mature MII oocytes and cumulus complexes were collected by flushing the separated oviducts with M199 medium (Sigma Chemical Company). After exposure to 300 IU/ml hyaluronidase (Sigma) in M199 for 3–5 min, cumulus cells were stripped from the oocyte by repeated gentle pipetting. Oocytes with intact first polar body and a finely granulated ooplasm were selected for subsequent treatments.

Enucleation of Recipient Oocytes

Telophase II enucleation were used for both goat oocytes and rabbit oocytes. Following IVM for 24 hr, about 40% caprine oocytes were spontaneously activated, with partially extruding second polar body in a membrane protrusion containing all the nuclear material. The membrane protrusion was obvious under an inverted microscope (Fig. 1B,C).

Selected rabbit oocytes were stimulated by double (1 sec apart) DC pulses of 1.2 KV/cm for 40 μsec in a fusion chamber containing 100 μl electrical fusion medium (0.25 M sorbitol, 0.5 mM Hepes, 0.1 mM calcium acetate and 0.5 mM magnesium acetate and 1 mg/ml of bovine serum albumin (BSA) in deionized water with an ECM2001 Electrocell Manipultor (BTX, San Diego, CA). Following stimulation, oocytes were washed three times in TCM199 supplemented with 10% FBS and then were incubated in TCM199 for 30 min. Activated rabbit oocytes with the first polar body, partially extruding second polar body and finely granulated ooplasm were selected for subsequent experiments (Fig. 2).

Before enucleation, all oocytes were incubated in TCM199 containing 10% FBS, 7.5 μg/ml Hoechst 33343, 7.5 μg/ml CB for 15 min. To enucleate rabbit oocytes, the first polar body or the partially extruding polar body together with small amount of underneath cytoplasm was aspirated using a 15–20 μm outer diameter glass

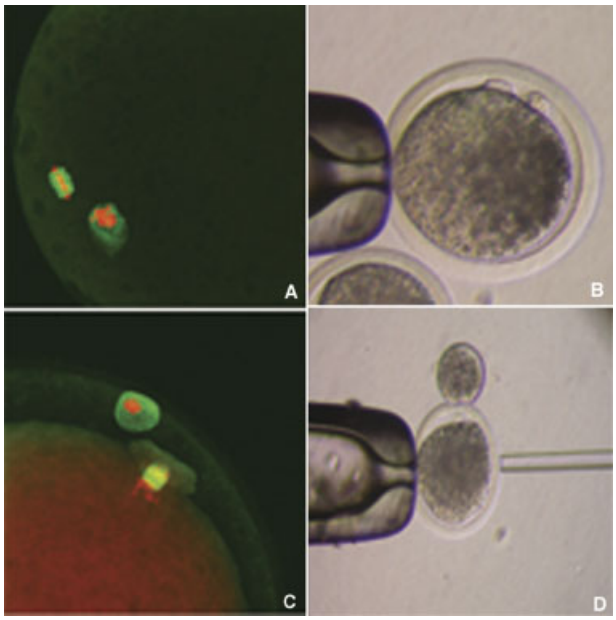

Fig. 1. Enucleation of goat oocytes at TII phase by pressing method. **A**: In vitro matured goat oocytes at MII. **B**: An oocyte with partially extruded second polar body under light microscopy. **C**: Confocal image of oocyte with partially extruded second polar body. **D**: Enucleation of goat oocytes at TII phase by pressing method.

micropipette. To enucleate caprine oocytes, a slit on the zona pellucida was made just over the membrane protrusion using a sharp needle and then the oocytes were pressed using injection pipette with blunt tip, pressing the membrane protrusion, first polar body, and a small part of adjacent cytoplasm out through the slit (Fig. 1D). The removed karyoplast was exposed to ultraviolet light to confirm the presence of nucleus. Only oocytes from which the chromosomes were entirely removed were used for nuclear transfer. In some experiments, MII-arrested goat oocytes and rabbit oocytes were enucleated by the same pressing method or aspiration method as described above, respectively.

Nuclear Transfer

After enucleation, all oocytes were immediately reconstructed. A micropipette containing the whole donor cell was introduced through the same slit of zona made during enucleation and the cell was inserted between the zona and the cytoplast membrane to facilitate close membrane contact and subsequent fusion. Reconstructed oocytes were equilibrated in fusion medium for 2 min. The couplets were then transferred into a fusion chamber containing 100 μl of fusion medium. The reconstructed embryos were manually aligned so that the contacting membrane of the cytoplast and donor cell was parallel to the electrodes. And then the couplets were electrically fused with an ECM2001 Electrocell Manipulator (BTX). Couplets were then washed in M199 supplemented with 10% FBS three times and incubated in the same medium for 30 min at 38°C in a humidified air containing 5% CO_2. Couplets were then checked for fusion under an inverted microscope.

Embryo Activation

The fused Tibetan antelope-goat embryos were chemically activated. They were first incubated in CR1aa containing 5 μM/L ionomycin for 5 min at RT, then they were incubated in CR1aa containing 2 mM/L 6-DMAP for 4 hr at 38.5°C under 5% CO_2 in humidified air. The ionomycin and 6-DMAP stocks were prepared in dimethyl sulfoxide (DMSO) and diluted to the desired concentration in CR1aa. The fused Tibetan antelope-rabbit embryos were activated by double DC pulses of 1.2 kV/cm for 40 μsec in the fusion medium. Activated couplets were extensively washed before transferring them into the culture droplets.

Embryo Culture In Vitro

All the activated Tibetan antelope-rabbit embryos were cultured in groups of around 20 embryos in 100 μl droplets of M199 supplemented with 10% FBS overlaid with mineral oil. Most activated Tibetan antelope-caprine embryos were cultured on monolayer of murine primary cell in 100 μl droplets of CR1aa overlaid with mineral oil to 2- to 4-cell stage and transferred to recipient goats to study the in vivo development. Small parts of Tibetan antelope-goat embryos were cultured in CR1aa droplets alone or cocultured with a layer of primary murine cell to study the in vitro development.

Embryo Transfer

Lubei white recipient does and Yimeng black recipient does were selected from those exhibiting a natural estrus 1 or 2 days prior to scheduled embryo transfer. Recipient does were fasted 24 hr prior to surgery. Anesthesia was induced immediately before surgery. Recipients were examined to confirm if they had one or more recent ovulations or corpora lutea present on the ovaries. Cleavage-stage embryos (2-cell to 4-cell stage) were transferred into the oviduct ipsilateral to the ovary with the most ovulation points. Recipients were returned to the herd to await subsequent examination of pregnancy status. Antibiotics and analgesics were administered according to approved procedures.

Confocal Microscopy

Samples were collected and fixed with 4% (w/v) paraformaldehyde in PBS (pH 7.4) for 40 min at RT. Fixed samples were permeabilized by transferring into PBS supplemented with 0.1% Triton X-100 and 0.3% BSA for 30–40 min (RT). After washing in PBS containing 0.01% Triton X-100, samples were incubated in blocking solution (PBS containing 1% BSA) for 1 hr at RT. The microtubules were localized by incubation for 1 hr at RT with a fluorescein isothiocyanate-labeled mouse monoclonal antibody against α-tubulin, which was diluted 1:100 in blocking solution. Nuclear status of samples was evaluated by staining with 10 μg/ml PI in PBS for 10 min. Following extensive washing, samples were mounted on slides with antifluorescence-fade medium (Sigma). Finally, the samples were observed under a Lecia Sigma confocal laser-scanning microscope (TCS-4D, Bensheim, Germany).

Statistical Analysis

Percentages of embryos at different stages were compared between groups using χ^2 analysis. Significant difference was determined at $P < 0.05$.

RESULTS

Collection and In Vitro Maturation Goat Oocytes

In the present study, a total of 13,387 abattoir-derived goat ovaries were collected and a total of 46,855 oocytes were obtained. Following IVM for 24 hr, 27,316 (58.3%) oocytes were matured and extruded the first polar body. Among matured oocytes 11,090 (40.6%) reached TII phase, with partially extruding second polar body (Fig. 1B,C).

Effect of Culture Conditions on In Vitro Development of Reconstructed Embryos

Activated Tibetan antelope-goat embryos were randomly selected and allocated to two culture systems. In group 1, cloned embryos were cultured in mCR1aa alone and 62.0% (62 of 100), 49.0% (49 of 100), 28.0% (28 of 100), 11.0% (11 of 100) reached 2-cell, 4-cell, 8-cell, and morula stage, respectively. However, none of the embryos could develop beyond morula stage. In group 2, cloned embryos were cocultured in mCR1aa droplets with monolayer of murine primary cell 66.4% (83 of 125), 52.0% (65 of 125), 43.2% (54 of 125), and 25.6% (32 of 125) embryos reached 2-cell, 4-cell, 8-cell, and morula stage, respectively. Four percent (5 of 125) developed to blastocyst, indicating that coculture is beneficial to overcome in vitro developmental block (Table 1) (Fig. 3).

Effect of Enucleation Timing on In Vitro Development of Cloned Embryos

Compared with cloned embryos using MII phase enucleated oocytes as recipient, slightly higher fusion rate and development rate were obtained for Tibetan antelope-goat embryos (73.9% vs. 67.4% and 4.0% vs. 1.1%, respectively) when using TII phase enucleated oocytes as recipients. Similarly, slightly higher fusion rate and development rate were obtained using TII phase enucleated rabbit oocytes as recipient than using MII phase enucleated rabbit oocytes as recipients (71.4% vs. 67.2% and 11.1% vs. 7.4%, respectively) (Tables 2 and 3) (Fig. 4).

Chromosome Analysis of Donor Cells at Different Passages

Chromosome analysis demonstrated that a majority of cells (83% of cells of passages 3–8 and 79% of cells of passages 10–17, respectively.) showed a normal chromosome complement (60 chromosomes) (Table 4). Figure 4

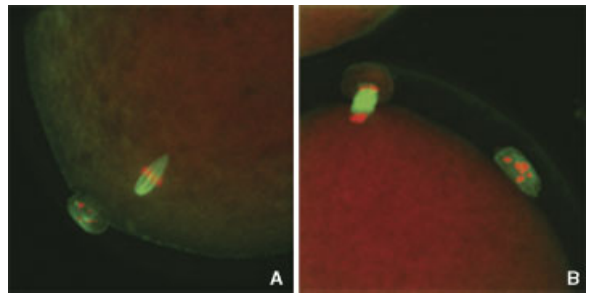

Fig. 2. Rabbit oocytes at MII phase and TII phase. **A**: Rabbit oocytes arrested at MII phase. **B**: Rabbit oocytes at TII phase activated by double DC.

Effect of Donor Cell Passages on Development of Tibetan Antelope-Goat Embryos

Significantly higher fusion rate and blastocyst rate were obtained from donor cells of passages 3–8 than that of passages 10–17 (77.8% vs. 61.3%, 2.9% vs. 0, respectively), suggested that long-term culture of donor cells decreased the fusibility and development potential of nuclear transfer embryos (Table 5).

Results of Embryo Transfer

A total of 2,397 cloned Tibetan antelope-caprine embryos at 2–4 stages were surgically transferred into oviducts of 66 surrogate does. A total of 51 does returned to estrous within 20 days after embryo transfer, 12 does returned to estrous between 30 and 40 days after embryo transfer, 2 does returned to estrous 60 days after embryo transfer, the last doe returned to estrous 78 days after embryo transfer. However, none of them developed to term.

DISCUSSION

Production of cloned animals by somatic cell nuclear transfer has been successful in many species. The efficiency of cloning is, however, very low for still unknown factors. Factors influencing the success are rather complex but in general they can be divided into two groups: (1) biological factors, including species-specific difference, the characteristics of karyoplast, and cytoplast interaction between them and (2) technical factors. The preparation of recipient cytoplast belongs to technical aspects and is a key factor that determines the overall efficiency of animal cloning (Jr et al., 2004).

TABLE 1. Effect of Culture Systems on the In Vitro Development of Tibetan Antelope-Goat Cloned Embryos

Culture type	Number of cultured embryos	2-cell number (%)	4-cell number (%)	8-cell number (%)	Morula number (%)	Blastocyst number (%)
CR1aa alone	100	62 (62.0%)[a]	49 (49.0%)[a]	28 (28.0%)[a]	11 (11.0%)[a]	0 (0%)[a]
Coculture	125	83 (66.4%)[a]	65 (52.0%)[a]	54 (43.2%)[b]	32 (25.6%)[b]	5 (4.0%)[b]

Values with different superscript within each column are significantly different (at least $P < 0.05$).

TABLE 2. Effect of Different Enucleation Timing on In Vitro Development of Tibetan Antelope-Goat Embryos

Enuleation timing	Number of NT units	Fused number (%)	Number of embryos cultured	Cleaved number (%)	Blastocyst number (%)
MII	187	126 (67.4%)[a]	89	51 (57.3%)[a]	1 (1.1%)[a]
TII	180	133 (73.9%)[a]	125	83 (66.4%)[a]	5 (4.0%)[a]

Values with same superscript within each column are not significantly different ($P > 0.05$).

Traditionally, cytoplasts are prepared by physical micromanipulation at MII phase. The first polar body and around 30% adjacent cytoplasm containing the metaphase plate are blindly aspirated or squazed out. Physical enucleation is technically demanding time-consuming, inherently invasive, and clearly damaging to cytoplast spatial organization and development of reconstructed embryos is inherently inefficient. As an alternative to MII phase enucleation, in the present study, activated oocytes were enucleated at TII phase. Compared with arrested MII oocyte-derived cytoplasts, the use of an activated TII cytoplasm may have several practical and biological advantages (Vilceu and Lawrence, 1998; Baguisi et al., 1999). Enucleation of TII oocytes is technically easier and removes considerably fewer cytoplasm (around 10%) and fewer cytoplasmic factors and organelles were lost compared with MII enucleation during which around 30% cytoplasm was removed. Moreover, TII enucleation provides a synchronous population of activated recipient cytoplasts that when used for embryo reconstruction show a higher rate of embryonic development in vitro. Results of the present study confirmed the beneficial effect of using TII enucleated oocytes as recipient. Higher fusion rate and development rate were obtained for both Tibetan antelope-goat and Tibetan antelope-rabbit embryos using TII phase enucleated oocytes as recipient. The smaller perivitelline space of TII enucleated oocytes and closer membrane contact between donor cell membrane and cytoplasm membrane may be one reason for higher fusion rate. More cytoplasmic factors and organelles present in TII enucleated oocytes may be beneficial for in vitro development.

In the present study, abattoir-derived and in vitro matured caprine oocytes contain many lipid granules and are opaque. The caprine plasma membrane is fragile and easy to break during enucleation. So, pressing method was adopted to enucleate caprine oocytes. The pressing method avoided piercing the plasma membrane directly by an aspirating pipette and ensured the minimal trauma to the oocytes.

In mammalian species, the maternal-zygotic transition (MZT) occurs at earlier embryonic stages and is often associated with embryo compaction and developmental blocks (Frei et al., 1989; Telford et al., 1990). This MZT, which is species-dependent and occurs at a particular stage of development, is characterized by a large increase in detectable transcription (Telford et al., 1990; Campbell, 1999). The completion of MZT leads the nucleus to take full control of the embryo development. For somatic nuclear transfer embryos, there is a similar transition associated with the nucleus reprogramming (Campbell, 1999). However, it was not clear whether the timing of this transition is recipient-specific or nucleus-specific for interspecies cloned embryos. The timing of MZT for both rabbit and goat occurs at 8- to 16-cell stage (Manes, 1973; Telford et al., 1990; Henrion et al., 1997). There is no information on early cleavage of Tibetan antelope embryo in vitro. In the present study, the MZT timing of the interspecies cloned Tibetan antelope embryos was investigated using two types of interspecies cloned embryos: Tibetan antelope-goat embryos and Tibetan antelope-rabbit embryos. Most Tibetan antelope-goat cloned embryos arrested at morula stage for around 2 days and then degraded. Only a small portion (4.0%) could develop further and reach blastocyst stage. The result suggested that there may be a morula-to-blastocyst block for normal Tibetan antelope embryos that is different from goat and the block of Tibetan antelope-goat cloned embryos maybe donor-specific.

However, different phenomenon was observed in Tibetan antelope-rabbit embryos. Most Tibetan antelope-rabbit cloned embryos arrested at 8-cell stage and subsequently degenerated when cultured in vitro. Only a small portion (11.1%) of embryos could develop to blastocysts, indicating there may be an 8- to 16-cell stage block which is same as rabbit embryos for Tibetan

TABLE 3. In Vitro Development of Tibetan Antelope-Rabbit Interspecies Cloned Embryos

Recipient type	Number of NT	Fused number (%)	2-cell number (%)	4-cell number (%)	8-cell number (%)	Morula number (%)	Blastocyst number (%)
MII enucleation	180	121 (67.2%)[a]	59 (48.8%)[a]	51 (42.1%)[a]	45 (37.2%)[a]	20 (16.5%)[a]	9 (7.4%)[a]
TII enucleation	126	90 (71.4%)[a]	46 (51.1%)[a]	39 (43.3%)[a]	35 (38.9%)[a]	16 (17.8%)[a]	10 (11.1%)[a]

Values with same superscript within each column are not significantly different ($P > 0.05$).

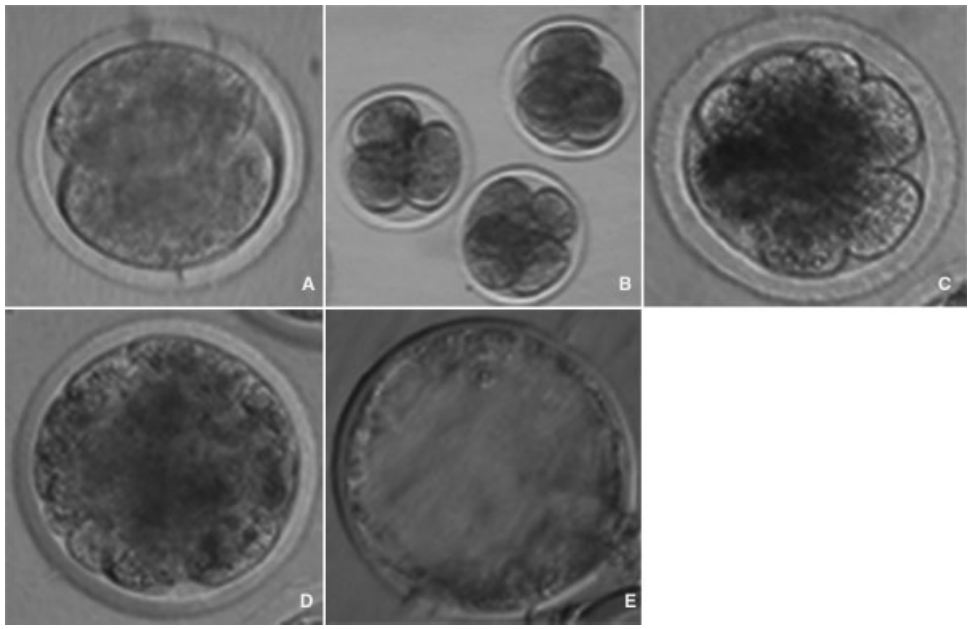

Fig. 3. Tibetan antelope-goat cloned embryos at different stages. **A**: Reconstructed embryo at the 2-cell stage (200×). **B**: Reconstructed embryo at the 4-cell stage (200×). **C**: Reconstructed embryo at the 8-cell stage (200×). **D**: Reconstructed embryo at the morula stage (200×). **E**: Reconstructed embryo at blastocyt stage (200×).

antelope-rabbit cloned embryos and the in vitro development block was recipient-specific.

Why the MZT timing is different for Tibetan antelope-goat and Tibetan antelope-rabbit embryos? Two explanations may be possible. One explanation is that MZT timing of interspecies cloned embryos is related to characteristics of oocyte recipient. Oocytes of different species may have different ability to reprogram somatic cells and to support development of interspecies cloned embryos. The more close the species of recipient oocyte is to the donor cell (goat vs. rabbit), the better the embryo development in vitro and the later the MZT.

Another explanation may be that chromosome number similarity and interactions between donor and recipient play an important role in determining MZT timing. Chromosome number could be related to in vitro developmental block found when interspecies nuclear transfer procedures were undertaken in species with dissimilar 2n chromosome number. In an interspecies cloning research, no in vitro developmental block was detected and interspecies cloned embryos reached blastocyst stage at high rate (Sansinena et al., 2005). Authors contributed the high development rate to the fact that both donor and recipient have same numbers of chromosome, implying that chromosome number could be related to the in vitro developmental block and similarity of chromosome numbers between donor and recipient may be beneficial for development. In the present study, both Tibetan antelope and goat have 30 pairs of chromosomes. The similarity of chromosome number between donor and recipient may be benificial for development, so the MZT timing is postponed and MZT is the result of combined effects of donor and recipient. It seems that the more similar the chromosome number of recipient oocyte is to donor species (goat vs. rabbit), the later the MZT occurs.

The true indication of successful reprogramming of somatic cells in nuclear transfer embryos is the production of live offspring. In order to further evaluate the developmental capacity of Tibetan antelope-goat embryos, embryos at 2- and 4-cell stage were surgically transferred into oviducts of 66 synchronized does. Fifty-one does returned to estrous within 20 days after embryo transfer, 12 does returned to estrous between 30 and 40 days after embryo transfer, two does returned to estrous 60 days after embryo transfer, and the last doe

TABLE 4. Chromosome Analysis of Donor Cells at Different Passages

Number of cell passages	Number of (%) cells with chromosomes of			Number of spreads counted
	<60	60	>60	
3–8	11 (9.0%)	105 (83.0%)	10 (8.0%)	126
10–17	16 (14.0%)	93 (79.0%)	9 (7.0%)	118

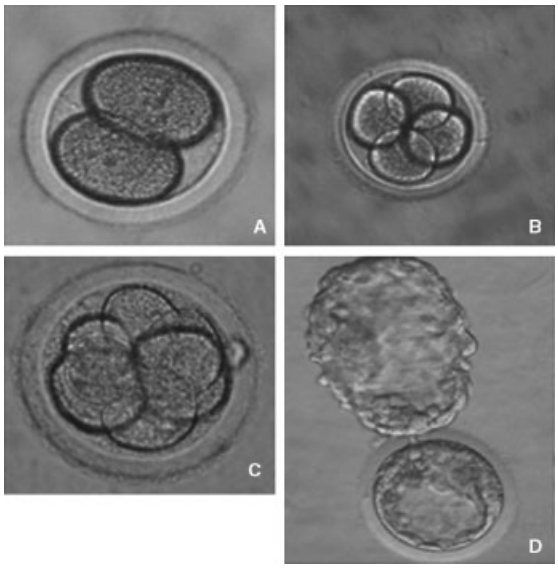

Fig. 4. Tibetan antelope-rabbit cloned embryos at different stages. **A**: Reconstructed embryo at the 2-cell stage (200×). **B**: Reconstructed embryo at the 4-cell stage (200×). **C**: Reconstructed embryo at the 8-cell stage (200×). **D**: Reconstructed embryo at blastocyt stage (200×).

returned to estrous 78 days after embryo transfer. Reasons for failure of full-term development is unclear. In our previous study, live and healthy cloned kids were obtained in Asian yellow goat cloning (unpublished data), so the embryo transfer technique should not be the main reason. During interspecies NT procedure, foreign mitochondria are introduced, mitochondria heteroplasmy may influence embryonic development and in uterine survivability. In previous interspecies NT research that has resulted in pregnancy, namely the gaur, the mouflon and the banteng cloning, donor cells and oocyte/surrogate are of same genus but different species. In the present study, donor cells and oocyte/surrogate are of different genus. They are far apart in their taxonomic classification than those in previous interspecies research. The physiological characteristics of Tibetan antelope are quite different from those of surrogate does used in the present study. To a certain extent, the physiological incompatibility may be a reason for failure of full-term development. Whether there is more suitable surrogate and whether these interspecies cloned embryos are able to develop to term in optimal uteri are questions that require further study.

TABLE 5. In Vitro Development Rate of Tibetan Antelope-Goat Embryos From Somatic Cells at Different Passages

Passage number	Number of NT units	Fused number (%)	Cleaved number (%)	Blastocyst number (%)
3–8	266	207 (77.8%)[a]	155 (74.9%)[a]	6 (2.9%)[a]
10–17	274	168 (61.3%)[b]	119 (70.8%)[a]	0[b]

Values with different superscript within each column are significantly different (at least $P < 0.05$).

In conclusion, to our knowledge, this is the first report of interspecies nuclear transfer using goat oocyte as recipient. Although no viable fetus was obtained, the present study demonstrates that Tibetan antelope fibroblast nuclei can be dedifferentiated in enucleated goat oocytes and these hybrid embryos can develop to blastocyst stage in vitro.

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