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RESEARCH ARTICLE

In vivo and *in vitro* development of Tibetan antelope (*Pantholops hodgsonii*) interspecific cloned embryos

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Abstract The Tibetan antelope is endemic to the Tibetan Plateau, China, and is now considered an endangered species. As a possible rescue strategy, the development of embryos constructed by interspecies somatic cell nuclear transfer (iSCNT) was examined. Tibetan antelope fibroblast cells were transferred into enucleated bovine, ovine and caprine oocytes. These cloned embryos were then cultured *in vitro* or in the oviducts of intermediate animals.

Less than 0.5% of the reconstructed antelope-bovine embryos cultured *in vitro* developed to the blastocyst stage. However, when the cloned antelope-bovine embryos were transferred to caprine oviducts, about 1.6% of the embryos developed to the blastocyst stage. In contrast, only 0.7% of the antelope-ovine embryos developed to the morula stage and none developed to blastocysts in ovine oviducts. The treatment of donor cells and bovine oocytes with trichostatin A did not improve the embryo development even when cultured in the oviducts of ovine and caprine. When the antelope-bovine embryos, constructed from oocytes treated with roscovitine or trichostatin A, were cultured in rabbit oviducts 2.3% and 14.3% developed to blastocysts, respectively. It is concluded that although some success was achieved with the protocols used, interspecies cloning of Tibetan antelope presents difficulties still to be overcome. The mechanisms resulting in the low embryo development need investigation and progress might require a deeper understanding of cellular reprogramming.

Keywords interspecific nuclear transfer, bovine, ovine, caprine, oviduct, apoptosis

1 Introduction

The Tibetan antelope (*Pantholops hodgsonii*) is the sole member of the genus *Pantholops*. It was formerly classified in the subfamily Antilopinae, but morphological and molecular evidence led to it being placed in its own subfamily, Pantholopinae, which is closely allied to caprine-antelopes of the subfamily Caprinae [1–3]. The Tibetan antelope inhabits open alpine and cold steppe environments between 3250 and 5500 m elevation. They are found almost entirely in China, where they inhabit Tibet, southern Xinjiang and western Qinghai and their numbers were nearly a million at the turn of the 20th century. Today, the majority are found within the Chang Tang Nature Reserve of northern Tibet and the numbers continue to drop yearly and were recorded at less than 75000 in 2008 [2–4]. Tibetan antelope wool, approximately 10 μm in diameter, is regarded as the best quality cashmere and is of great economic value [5]. Tibetan antelope are listed as endangered by the World Conservation Union, a consequence of commercial poaching for their underwool and competition with local domesticated herds.

Rescue and conservation of this unique species is now an important concern of biologists and ecologists. One recent innovative technique, somatic cell nuclear transfer (SCNT), has been applied to the transfer of wildlife or endangered animal somatic cells to domestic animal oocyte cytoplasts to create interspecies (iSCNT) cloned embryos. The iSCNT studies have been reported in endangered animals including giant panda (*Ailuropoda melanoleuca*) [6,7], Tibetan antelope (*Pantholops hodgsonii*) [8], black bear (*Ursus thibetanus*) [9], argali (*Ovis ammon*) [10], banteng (*Bos javanicus*) [11], antarctic minke whale (*Balaenptera bonaeresis*) [12], desert bighorn ovine (*Ovis canadensis*) [13], yak (*Bos grunniens*) [14], takin (*Budorcas taxicolor*) [15], siberian tiger

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(*Panthera tigris altaica*) [16], gianteland (*Taurotragus oryx*) [17], red panda (*Ailurus fulgens*) [18], sei whale (*Balaenoptera borealis*) [19]. However, the iSCNT cloned embryos of these animals developed to blastocyst stage at discouragingly low percentages. The best results of iSCNT in mammals were obtained from the subspecies and sibling species that can hybridize naturally as cloned garu (*Bos gaurus*) [20], argali (*Ovis ammon*) [10], and river buffalo (*Bubalus bubalus arnee*) [21].

In the present study, systematic integrated protocols including the choice of different recipient cytoplasts of bovine, ovine and caprine, culture of the cloned embryos *in vitro* or *in vivo* in oviducts of live animals, and combinations with cellular reprogramming associated small molecules were evaluated for iSCNT embryo development. The fibroblast cells derived from a Tibetan antelope were transferred into enucleated bovine, ovine and caprine oocytes. The iSCNT embryos were treated with trichostatin A (TSA), a specific histone deacetylase inhibitor, or roscovitine (ROS), a cell cycle dependent protein kinase inhibitor, and then cultured *in vitro* or in the oviducts of ovine, caprine and rabbit.

2 Materials and methods

All the chemicals were obtained from Sigma Chemical Co. (St Louis, MO, USA), unless otherwise stated.

2.1 Establishment of donor cell line

A piece of ear tissue was obtained from a two-year old male Tibetan antelope from Qinghai Wildlife Zoo Park in Xining with the permission from the Forestry Bureau, Qinghai Province. The biopsy specimen was washed and cut into small pieces in phosphate-buffered saline (PBS). Tissue explants were seeded into 35-mm dishes and incubated at 37°C for 3 h. When the explants had firmly adhered to the surface, they were cultured in 2 mL of Dulbecco's modified Eagle's medium/F-12 (DMEM/F-12, Gibco) supplemented with 20% fetal calf serum (Gibco) in 5% CO_2 at 37°C. After the cells reached 90% confluence, they were harvested by trypsinization (Gibco) and seeded in three 35-mm dishes. The cells from passage numbers 3–9 were used as nuclear donors.

2.2 Karyotype analysis

Karyotypes of Tibetan antelope, bovine, ovine and caprine cell lines were prepared by air-dried method and analyzed as reported [22]. Briefly, the cells were treated with colcemid and then trypsinized and centrifuged. After treatment with 0.075 $mol \cdot L^{-1}$ KCl hypotonic solution, the samples were fixed with 3:1 methanol:glacial acetic acid and the resuspended fixed cells were placed on clear slides and air-dried. The slides were stained with Geimsa and observed under light microscopy and photographed.

2.3 Maturation and preparation of recipient oocytes

Bovine, ovine and caprine ovaries were collected from the local slaughterhouses, and transported to the laboratory in saline within 2 h of collection. For ovine or caprine ovaries, the cumulus oocyte complexes (COCs) were released from the follicles by cutting the ovaries with sharp bistouries in Dulbecco's PBS (DPBS) containing 1% fetal bovine serum (FBS) and 10 $mg \cdot mL^{-1}$ heparin as described in our previous reports [23,24]. The bovine COCs were aspirated from 2 to 8 mm follicles and cultured in maturation medium (M199, Gibco) supplemented with 10% FBS (Gibco), 0.01 $IU \cdot mL^{-1}$ follicle-stimulating hormone (FSH), 1 $IU \cdot mL^{-1}$ luteinizing hormone (LH), 0.01 $\mu g \cdot mL^{-1}$ estradiol, 100 $U \cdot mL^{-1}$ penicillin and 100 mg $\cdot mL^{-1}$ streptomycin. A total of 50–60 COCs were transferred in 500 μL maturation medium in a 4-well dish. The COCs were cultured at 38.5°C in a humidified 5% CO_2 in air atmosphere for 18 h. After maturation, the cumulus cells were completely removed by vortexing the COCs in 1 $mg \cdot mL^{-1}$ hyaluronidase. Oocytes with the first polar body (PB1) and identical cytoplasts were selected as recipient oocytes for the nuclear transfer.

2.4 Nuclear transfer, fusion and activation

SCNT protocols used in this experiment were as previously reported [25,26]. Briefly, the selected oocytes were placed into a 30 μL microdrop of M199 medium (Gibco) containing 1% FBS, 7.5 $\mu g \cdot mL^{-1}$ cytochalasin B and 7.5 $\mu g \cdot mL^{-1}$ Hoechst 33342 covered by mineral oil. The PB1 and the adjacent cytoplast containing oocyte chromosomes were removed by a glass pipette (18–20 μm) under 200 × magnification. The enucleation process was performed under ultraviolet light to ensure removal of the oocyte chromatins. A single cell was placed into the perivitelline space of the enucleated oocytes. The cell-cytoplast complexes were placed in SOFaa containing 0.5% bovine serum albumin (BSA) and allowed to recover for 30 min.

The reconstructed couplets were electrically fused with an ECM 2001 Electro cell Manipulator (BTX), and incubated in SOFaa containing 0.5% BSA for 30 min at 38.5°C in a humidified atmosphere containing 5% CO_2. The fused embryos were chemically activated by incubation with 5 $\mu mol \cdot L^{-1}$ ionomycin for 5 min followed by incubation with 10 $\mu g \cdot mL^{-1}$ cycloheximide (CHX) for 5 h at 38.5°C in a humidified atmosphere containing 5% CO_2.

2.5 Embryo culture *in vitro*

Activated antelope-bovine embryos were cultured in SOFaa plus 0.5% BSA for 48 h and then checked for cleavage. The cleaved embryos were then continued to culture in SOFaa containing 4% FBS and incubated for further 5 d at 38.5°C in a humidified atmosphere containing 5% CO_2. The medium was replaced with fresh medium every 2 d. And we put bovine-bovine and ovine-ovine embryos as controls.

2.6 Culture of the cloned embryos *in vivo* in the oviducts of intermediate animals

Because of the low developmental rate of interspecies cloned embryos cultured *in vitro*, ovine, caprine and rabbit were used as intermediate animals for embryo culture, respectively. The fused cloned embryos were surgically transferred to the oviducts of the recipient animals, the embryos were then recovered from the oviducts or uterus 5 d later to observe the embryo development.

2.7 Oocytes treatment with TSA or ROS

Ovine and caprine COCs were incubated in the presence of TSA at 1.0 ng·mL^{-1} for 24 h to examine the effect on cloned embryo development. The treated oocytes were used for nuclear transfer and the resultant antelope-ovine and antelope-caprine embryos transferred to ovine and caprine oviducts, respectively.

The bovine COCs were treated with ROS for 5 h, and then incubated in normal maturation medium for further 17–18 h. A different group of COCs were treated with TSA at 1.0 ng·mL^{-1} for 24 h. The cloned embryos were transferred to the rabbit oviducts and recovered 5 d later. Untreated antelope-bovine embryos cultured in rabbit oviducts as control.

2.8 Apoptosis in the cloned embryos examined by TUNEL

DNA degradation in nuclei was detected using a cell death detection technique based on terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) with fluorescein-conjugated dUTP as reported [27] with minor modifications. The blastocysts were fixed in 4% paraformaldehyde, rinsed in PBS and then permeabilized in PBS with 0.1% Triton X-100 for 180 min and incubated in fluorescein-conjugated dUTP in the dark at 37°C for 1 h. The blastocysts were then counterstained with 50 μg·mL^{-1} propidium iodide for 5 min and observed under a fluorescence microscope.

2.9 Statistical analyses

The proportional data for embryo development were analyzed by the χ^2 test, and a P value of less than 0.05 was considered statistically significant.

3 Results

3.1 Chromosome analysis of Tibetan antelope, bovine, ovine and caprine

Chromosome analysis showed that the majority of the antelope cells (64.9% in passage 9 and 58.2% in passage 14) were with normal chromosomal composition ($2n = 60$). When compare with other animals the chromosomal complements of Tibetan antelope, bovine, caprine and ovine are 60, 60, 60 and 54 chromosomes, respectively. The chromosomes of the Tibetan antelope are telocentric (Fig. 1).

3.2 Development *in vitro* of the interspecific cloned embryos

The cleavage rates of the cloned antelope-bovine and antelope-ovine embryos were 56% and 64%, respectively, which were significantly lower than those of intraspecific bovine-bovine (75%) and ovine-ovine (81%) cloned embryos. In antelope-bovine, about 0.5% of the cloned embryos developed to the blastocyst stage. In antelope-ovine, however, although around 52% of the embryos developed to 8- to 16-cell stage none of the embryos developed to the morula stage (Fig. 2). All of the embryos ceased development at 8- to 16-cell stage. The blastocyst development rates in bovine-bovine and ovine-ovine were 31.9% and 11.8%, respectively (Table 1).

3.3 Development of the interspecific cloned embryos *in vivo* in intermediate animal oviducts

After transfer and incubation of the cloned embryos in ovine and caprine oviducts for 5 d, the recovery rates and embryo development were summarized in Table 2. Only 30% to 60% of the embryos were recovered. The percentage of the blastocyst development in antelope-bovine was 1.6%, but only 0.7% of the antelope-ovine embryos developed to the morula stage and no blastocysts were recovered (Fig. 2).

3.4 The *in vivo* development of the cloned embryos derived from TSA- or ROS-treated oocytes

After incubation of the antelope-bovine embryos derived from the ROS- or TSA-treated oocytes into rabbit oviducts for 5 d, 2.3% and 14.3% of the embryos had developed to the blastocyst stage, respectively. Unexpectedly, none of the antelope-caprine or antelope-ovine embryos developed to blastocyst stage (Table 3).

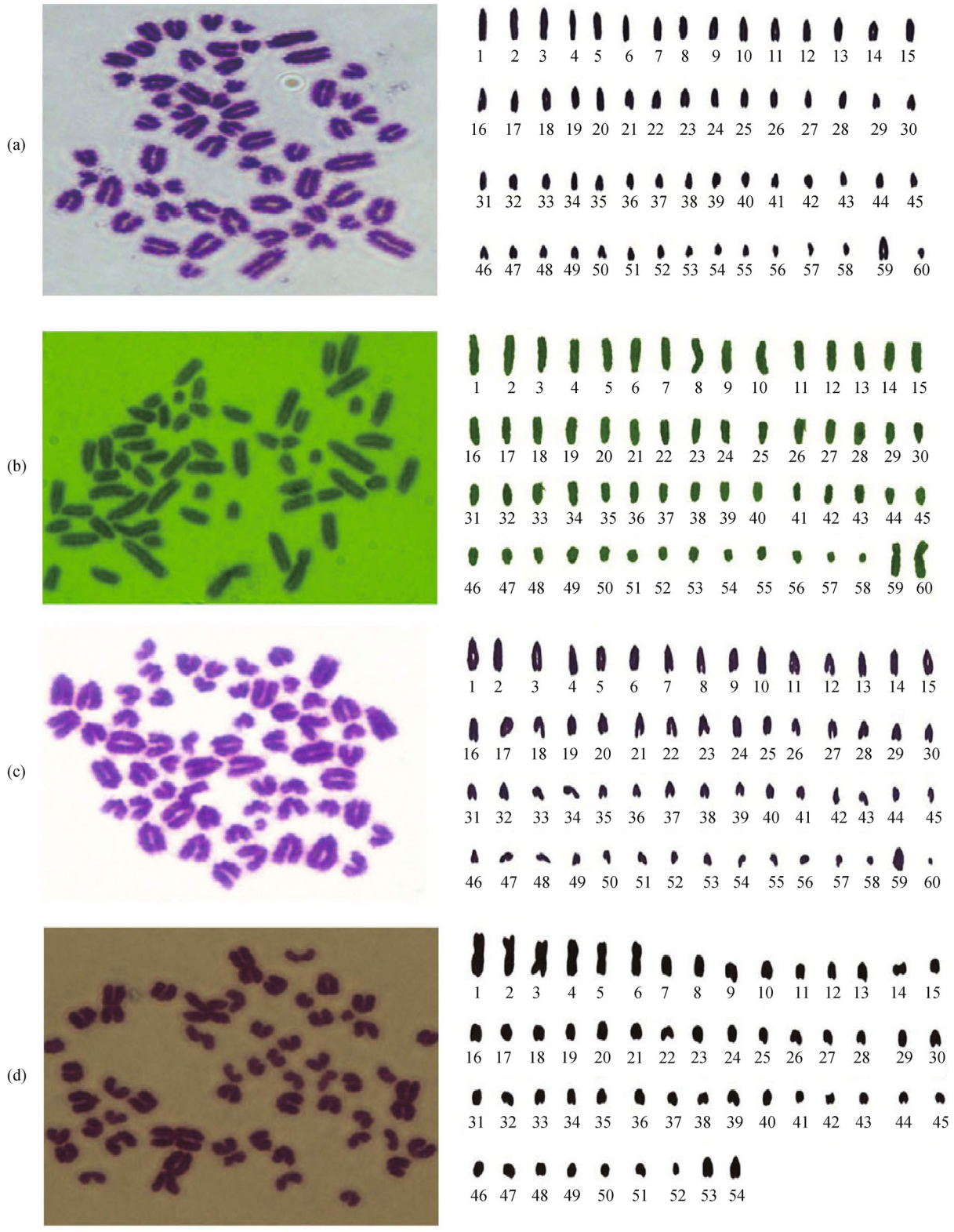

Fig. 1 Karyotype analyses of Tibetan antelope, bovine, caprine and ovine. (a) Tibetan antelope, $2n = 60$; (b) bovine, $2n = 60$; (c) caprine, $2n = 60$; and (d) ovine, $2n = 54$. (× 1000).

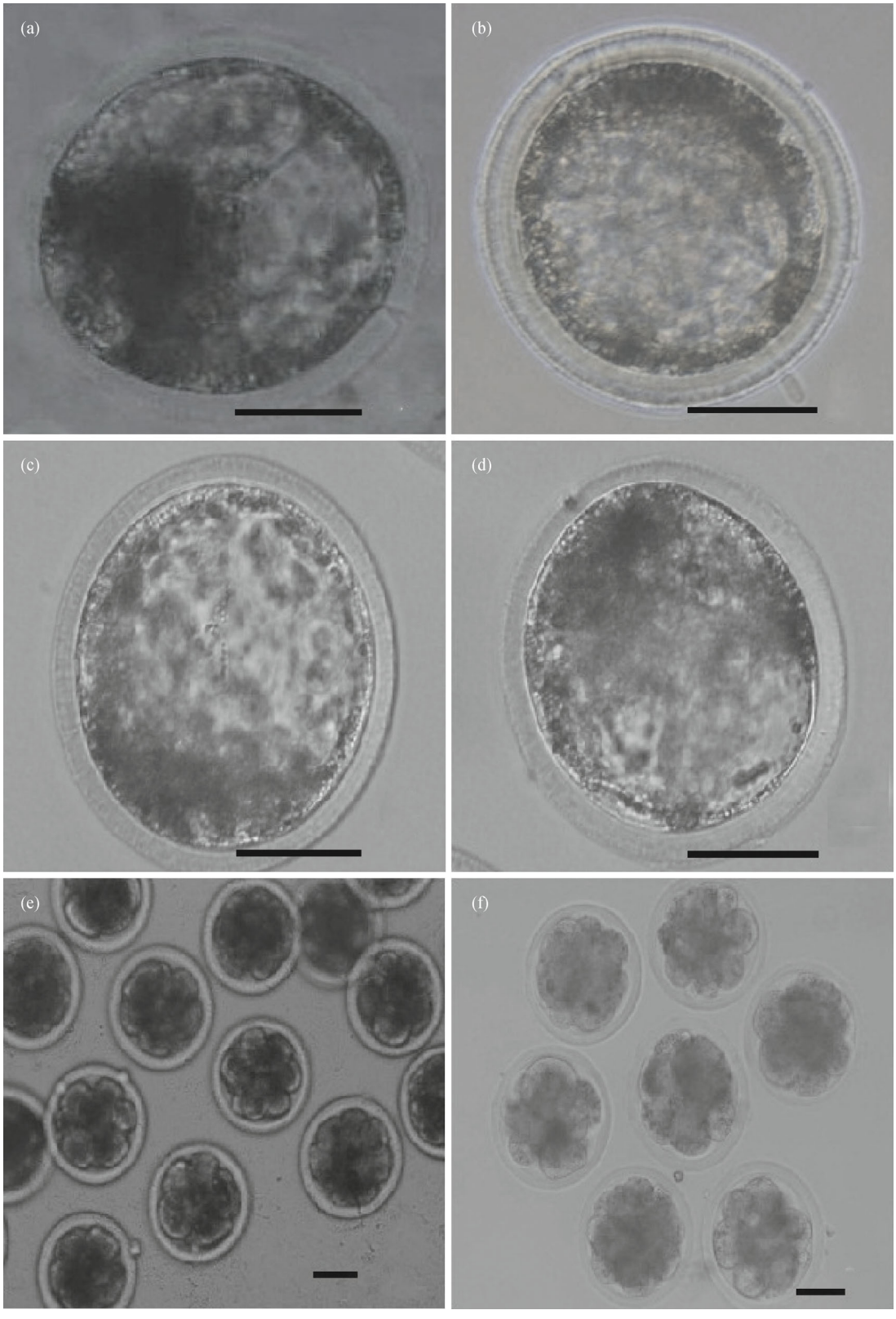

Fig. 2 Representative images of the Tibetan antelope interspecific SCNT embryos cultured *in vitro* or *in vivo*. (a) Antelope-bovine blastocyst derived from roscovitine-treated oocytes and incubated in rabbit oviduct; (b) antelope-bovine blastocyst derived from TSA-treated oocytes and incubated in rabbit oviduct; (c) antelope-bovine blastocyst incubated in caprine oviduct; (d) blastocyst obtained from *in vitro* culture; (e) 8- to 16-cell stage antelope-ovine cloned embryos cultured *in vitro*; (f) 8- to 16-cell stage antelope-bovine cloned embryos cultured *in vitro*. Each scale bar represents 100 μm.

3.5 TUNEL assay of the *in vitro* and *in vivo* developed embryos

The result showed that the apoptotic level of the *in vitro* developed cloned embryos was higher than that in embryos developed *in vivo*. The signals were detected at the 8- to 16-cell stage in *in vitro* developed embryos and at the morula stage *in vivo* developed embryos (Fig. 3).

Table 1 The *in vitro* development of the Tibetan antelope interspecific cloned embryos

Donor cells	Oocytes	Replicates	Embryos cultured	Embryos cleaved/%	8- to 16-cell embryos/%	Morulae/%	Blastocysts/%
Antelope	Bovine	9	420	234 (55.7)[a]	82 (19.5)[a]	3 (0.7)[a]	2 (0.5)[a]
Antelope	Ovine	7	236	152 (64.4)[a]	122 (51.7)[c]	0	0
Bovine	Bovine	5	257	168 (75.1)[b]	142 (55.3)[c]	112 (43.6)[c]	82 (31.9)[c]
Ovine	Ovine	6	195	158 (81.0)[b]	59 (30.1)[b]	28 (14.3)[a]	23 (11.8)[a]

Note: In the same column, values with different superscripts are significantly different, a:b, b:c, $P < 0.05$; a:c, $P < 0.01$.

Table 2 The *in vivo* development in intermediate animal oviducts of the Tibetan antelope interspecific cloned embryos

Oocytes	Intermediate animals	Replicates	Embryos fused/%	Embryos transferred	Embryos recovered/%	Over 2-cell/%	8- cell embryos/%	Morulae/%	Blastocysts/%
Ovine	Ovine	7	435 (81.9)	429	130 (30.3)[a]	31 (23.8)[a]	4 (3.1)[a]	1 (0.7)[a]	0
Bovine	Ovine	3	38 (79.1)	35	20 (57.1)[a]	10 (50.0)[b]	2 (10.0)[a]	0	0
Bovine	Caprine	4	178 (81.3)	168	62 (36.9)[a]	16 (25.8)[a]	5 (8.1)[a]	1 (1.6)[a]	1 (1.6)

Note: In the same column, values with different superscripts are significantly different, a:b, b:c, $P < 0.05$; a:c, $P < 0.01$.

Table 3 Oviduct i*n vivo* development of Tibetan antelope interspecific embryos from ROS- and TSA-treated oocytes

Treatments	Oocytes	Intermediate animals	Replicates	Cleaved/%	Embryos transferred	Embryos recovered /%	Morulae/%	Blastocysts/%
ROS	Bovine	Rabbit	4	136 (52.9)	136	43 (31.7)[a]	0	1 (2.3)[a]
TSA	Bovine	Rabbit	3	58 (58.6)	58	7 (12.1)[b]	0	1 (14.3)[a]
TSA	Ovine	Ovine	3	22 (60.1)	22	4 (18.2)[b]	2 (50.0)	0
TSA	Caprine	Caprine	3	72 (52.8)	72	19 (25.3)[ab]	0	0
Untreated control	Bovine	Rabbit	4	128 (54.0)	128	49 (38.3)[a]	0	0

Note: In the same column, values with different superscripts are significantly different, a:b, b:c, $P < 0.05$; a:c, $P < 0.01$.

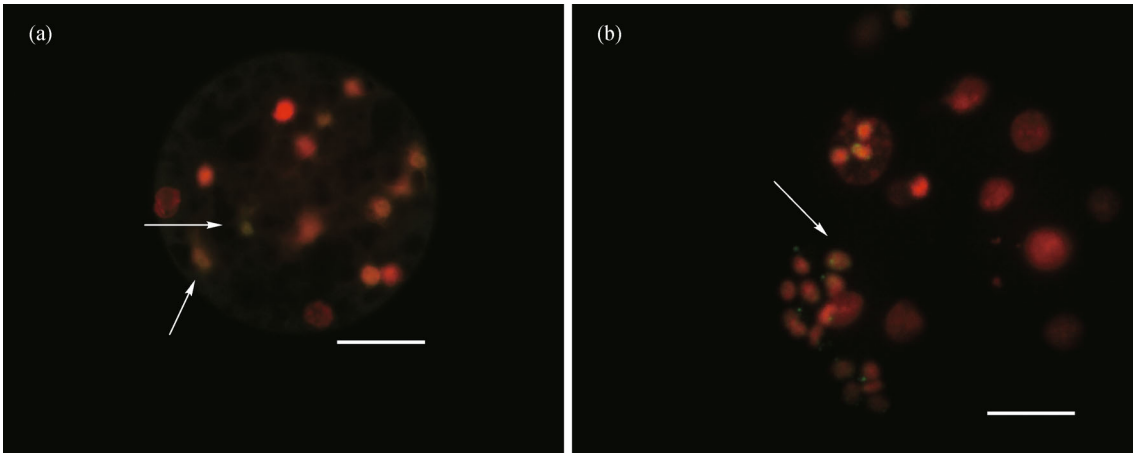

Fig. 3 Apoptosis in interspecific cloned embryos. (a) Antelope-bovine morulae cultured *in vitro*; (b) antelope-bovine morulae cultured *in vivo*. TUNEL stain or apoptosis appears green and nucleic acid appears red. The arrows indicate the sites of apoptosis. Each scale bar represents 100 μm.

4 Discussion

Tibetan antelope, bovine, ovine and caprine belong to Bovidae, but not in the same subfamily. Chromosomal analyses indicate that Tibetan antelope, bovine and caprine have 60 chromosomes, and ovine have 54. Consequently, bovine and caprine oocytes are probably a suitable choice for Tibetan antelope iSCNT. In a previous study, Zhao et al. [28] transferred antelope cells into rabbit enucleated oocytes and found that 1.4% to 8.7% of cloned embryos developed to blastocysts. When caprine oocytes were used as the recipient, around 2.9% of the cloned embryos developed to the blastocyst stage, and the *in vitro* developmental block occurred at the morula stage [8]. In the present study, the enucleation process was performed under ultraviolet light to ensure removal of the oocyte chromatins. We used ovine and bovine oocytes as the recipient cytoplasts and found that the cleavage rates of the cloned antelope-bovine and antelope-ovine embryos did not show any differences during *in vitro* development (56% and 64%, respectively). However, these percentages were lower than those obtained for the intraspecific cloned embryos of bovine and ovine (75% and 81%, respectively). Interestingly, we observed that more antelope-ovine cloned embryos (51.7%) reached the 8- to 16-cell stage than antelope-bovine embryos (19.5%) and intraspecific ovine embryos (30%). However, none of the cloned antelope-ovine embryos developed beyond the morula stage, which suggests that a developmental block occurred at morula stage, which is consistent with the previous finding [8]. In the antelope-bovine clones, a high proportion of the embryo development ceased the at 8- to 16-cell stage, indicating a block occurred at the 8- to 16-stage. When ovine, pig, monkey and rat cells were respectively transferred into enucleated bovine oocytes, the cloned embryos could develop to blastocysts with low percentages [29]. When transfer of argali (*Ovis ammon*) cell nuclei into the enucleated oocytes of ovine (*Ovis aries*) the iSCNT embryos developed to blastocysts [10]. Chen et al. [30] reported that transfer of skeletal muscle cells, uterine epithelial cells or udder cells of giant pandas into enucleated rabbit oocytes resulted in the cloned embryos developing to blastocysts. Transfer of takin and yak somatic cells into enucleated bovine oocytes resulted in blastocyst development [15]. Human somatic cells can be partially reprogrammed in enucleated bovine oocytes and the blastocyst formation rate is significantly lower than that of bovine-bovine cloned embryos [31]. These results suggest that iSCNT embryos can develop to morula/blastocyst stage but the majority of the embryos arrest at 8- to 16-cell stage.

Because of the low efficiency of the *in vitro* development, we designed the *in vivo* oviduct culture experiment. Theoretically, the oviducts and uterus are the optimal environment for embryo development. We selected the Bovidae animals caprine and ovine, and the laboratory animal rabbit as intermediates for *in vivo* culture. The antelope-bovine, antelope-caprine and antelope-ovine embryos were transfered into the oviducts of homologous recipient animals. Unfortunately, the blastocyst development of the cloned antelope embryos did not improve. Only 0.7% of the antelope-ovine embryos developed to the morula stage, and 1.6% of the antelope-bovine embryos developed to the blastocyst stage. The method from both the *in vitro* and *in vivo* incubation of antelope iSCNT embryos derived from bovine, ovine or caprine oocytes did not overcome the developmental block and the rate of cloned embryo development did not improve. The intermediate oviduct environment probably is not a suitable choice for antelope iSCNT embryo development. These results indicate that the interspecific antelope cloning has complications and difficulties that need further research to resolve.

TSA as a specific histone deacetylase inhibitor has been shown to improve SCNT embryo development [32]. In mouse, TSA treatment caused an increase in chromosome decondensation and nuclear volume in SCNT-generated embryos similar to that in embryos produced by intracytoplasmic sperm injection and histone acetylation increased in parallel with chromosome decondensation [33]. In cat-bovine, treatment of the cloned embryos with TSA resulted in significantly higher rates of cleavage and blastocyst formation (84.3% and 4.6%, respectively) than the non-TSA-treated embryos (63.8% and 0%, respectively) [34]. In the present study, TSA was used to treat both antelope cells and recipient ovine or caprine oocytes. After incubation of the antelope-caprine embryos in oviducts, a total of 10.5% of the embryos developed to the morula stage, but none of the embryos developed to the blastocyst. Disappointingly, the antelope-ovine embryos did not develop beyond the 8-cell stage. However, the TSA-treated bovine oocytes improved the antelope-bovine embryo development *in vivo* with 14.3% blastocyst development, but the sample was small. Roscovitine is a cell cycle dependent protein kinase inhibitor, which has been shown capable of reversibly inhibiting meiotic resumption in bovine oocytes for 24 h with no negative effects on subsequent development. It could make the cell cycle synchronization, and is advantageous to the oocyte maturation [35]. In this study, the ROS-treated bovine oocytes resulted in 2.3% blastocyst formation in rabbit oviducts. In summary, these results indicate that small molecules associated cellular reprogramming and the oviduct *in vivo* culture environment have somewhat beneficial effect on the antelope iSCNT embryo development, but the blastocyst formation is still extremely low.

The data presented here and in previous studies, indicate that iSCNT efficiency remains low despite the range of methods tried. The birth of gaur [20] and river buffalo [21] are contributed to the close relationship of donor cell and recipient cytoplasm either between subspecies or sibling

species. The biggest obstacle is probably the incompatibility between the donor nucleus and the recipient cytoplasm. It is important for the physiologic function of recipient mitochondria to coordinate with that of donor nuclei [36]. Wen et al. [37] demonstrated that the mtDNA of oocytes coexists with the mtDNA of donor cells before implantation in reconstructed panda-rabbit embryos. After implantation, however, donor cell mtDNA increased significantly, while the mtDNA of oocytes decreased markedly. Song et al. [38] believed that the content and expression quantity of donor cell mtDNA will increase gradually with early gene activation of the donor nucleus. Meanwhile, the amount of mitochondria in recipient oocytes will selectively decrease. In our unpublished work on the interspecies cloning of Przewalski's gazelle and bovine, the upregulated genes associated with nuclear reprogramming, the degradation maternal genes, and genes responsible to mtDNA functions are with significant differences between inter- and intra-specific cloned embryos.

5 Conclusions

The reconstructed Tibetan antelope-bovine embryos could develop to the blastocyst stage while the antelope-ovine and antelope-caprine cloned embryos did not develop beyond 8-cell or morula stage. When rabbit oviducts were used for intermediate *in vivo* culture, the antelope-bovine embryos exhibited better development. The present results revealed that interspecies Tibetan antelope cloning would be difficult to overcome at current situation. The mechanisms involved in the extremely low embryo development probably need much more efforts and more deep understanding of cellular reprogramming.

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Compliance with ethics guidelines Guanghua Su, Lei Cheng, Yu Gao, Kun Liu, Zhuying Wei, Chunling Bai, Fengxia Yin, Li Gao, Guangpeng Li and Shorgan Bou declare that they have no conflict of interest or financial conflicts to disclose.

All applicable institutional and national guidelines for the care and use of animals were followed.

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