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Effect of Mitochondrial Dysfunction in Nuclear Donor and Ooplasmic Recipient on Early Development of Goat-Sheep Interspecies Somatic Nuclear Transfer Embryos

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Abstract: Mitochondria is a kind of important organelle for producing and supplying energy in eukaryotes. The interspecies somatic nuclear transfer could result in coexisting of nuclear donor and ooplasmic recipient mitochondria in reconstructed embryos. In the present study, after nuclear donor and/or ooplasmic recipient mitochondria damaged by photoirradiating rhodamine-123, examination of the goat-sheep interspecies somatic nuclear transfer embryos early development ability *in vitro* was performed. The result shows that sheep ooplasmic recipient mitochondria damaged could result in reconstructed embryos development ability declined in 2-cell and morulae/blastocyst stage but does not affect 2-cells stage to 8-cells stage development; goat nuclear donor cells mitochondria damaged can not affect reconstructed embryos development ability before morulae/blastocyst stage; after both nuclear donor and ooplasmic recipient mitochondria are damaged, the reconstructed embryos development pattern is same as that of only ooplasmic recipient mitochondrial damaged. The result indicated that before interspecies somatic nuclear transfer reconstructed embryo development to morulae/blastocyst stage, ooplasmic recipient mitochondrial is dominant over nuclear donor mitochondrial in affecting reconstructed embryos development ability.

Key words: Mitochondria, interspecies somatic nuclear transfer, goat, sheep, donor cells, China

INTRODUCTION

In the mammal normal fertilization embryos, a kind of nuclear-encoded proteinaceous component of the sperm mitochondrial membrane was recognized and marked by a kind of proteolytic peptide-Ubiquitin then sperm mitochondria was destroyed by proteolysis in oocyte lysosome and proteasome (Sutovsky *et al*., 2000). This mechanism makes selection degeneration paternal mitochondria become possibly and causes mitochondria and mitochondrial DNA inherited exclusively from maternal. Nuclear transfer inevitable can introduced all or part nuclear donor mitochondria into enucleated oocyte. Nuclear donor mitochondria also can be replicated during nuclear transfer reconstructed embryos development (Meirelles *et al*., 2001). Thus, causes nuclear transfer reconstructed embryos even offspring have two kinds of mitochondria derived from nuclear donor and ooplasmic recipient.

Mature mammalian oocytes are maternally endowed with thousands of nonreplicating mitochondria that act as the founding population of all daughter-cell mitochondria of the developing embryos (Jansen, 2000; Van Blerkom *et al*., 2002). Mutational oocytes mtDNA may pass to daughter-cell due to lack of redundant templates or active repair mechanisms and cause embryos development arrest (Barrittm *et al*., 2002). Mitochondrial dysfunction in mouse oocytes can interfered significantly with the oocytes maturation process and result in preimplantation embryos arrest *in vitro* (Thouas *et al*., 2004, 2006; Takeuchi *et al*., 2005). In human, oocytes mitochondria activity declines correlate with the progressive age-related and results in low embryos development and pregnancy ability (Wilding *et al*., 2001). The destiny of nuclear donor mitochondria coming into ooplasmic recipient by nuclear transfer is different according to the donor cell type, the nuclear transfer procedure, nuclear donor and ooplasmic recipient

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different combination way (Ma *et al*., 2006). Many researches about mitochondrial origin and proportion in homogeneous and heterogeneous somatic nuclear transfer embryos and offspring were performed (Evans *et al*., 1999; Chen *et al*., 2002; Takeda *et al*., 1999, 2003; Hiendleder *et al*., 2003; Lanza *et al*., 2000; Loi *et al*., 2001; Steinborn *et al*., 1998, 2002; Yang *et al*., 2004) but report about the effect of nuclear donor and ooplasmic recipient mitochondria on interspecies nuclear transfer embryos development ability has not seen until now. In the present study, nuclear donor and/or ooplasmic recipient mitochondria was pre-damaged by photosensibilization then research the effect of mitochondrial dysfunction on goat-sheep interspecies somatic nuclear transfer embryos early development ability *in vitro*.

MATERIALS AND METHODS

Chemicals and medium: Unless otherwise indicated, all chemicals were purchased from Sigma (Sigma-Aldrich China Inc.) Rhodamine-123 (ROD-123), Gentamycin Sulfate; Fetal Bovine Serum (FBS; Hylone); Heparin sodium salt; Hyaluronidase; Dulbecco Modified Eagle Medium (DMEM, Gibco); trypsin (Amresio); mineral oil; Cytochalasin B (CB); Hoechst 33342; ionomycin; 6-Dimethylaminopurine (6-DMAP); Bovine Serum Albumin (BSA); Human Menopausal Gonadotropin (HMG; Serono); Epidermal Growth Factor (EGF; Gibco); Dimethyl Sulfoxide (DMSO); Tissue Culture Medium-199 (TCM-199; Gibco); Essential Amino Acid (EAA; Gibco); Nonessential Amino Acids (NEAA; Gibco); some kinds of inorganic salt. Oocyte Mature (OM) medium was TCM-199 supplemented with 10 mM Hepes; 0.38 mM sodium pyruvate; 50 μg mL^{-1} gentamycin sulfate; 10% (v/v) FBS; 1 μg mL^{-1} 17 β-estradiol; 0.075 IU mL^{-1} HMG; 10 ng mL^{-1} EGF. SOFaa medium is modified Synthetic Oviduct Fluid (SOF) medium supplemented with 2% (v/v) EAA; 1% (v/v) NEAA; 4 g L^{-1} BSA as described earlier (Gardner *et al*., 1994). Mitochongdrial stain medium is PBS medium supplemented with 5% (v/v) FBS; 50 μg mL^{-1} the mitochondrion-specific fluorophore ROD-123 prepared from a stock solution of 100 mg mL^{-1} ROD-123 in DMSO as described earlier (Thouas *et al*., 2004) and modified. Handling medium is PBS medium supplemented with 5 μg mL^{-1} CB, 5 μg mL^{-1} Hoechst 33342, 5% (v/v) FBS.

Sheep oocyte maturation *in vitro*: Slaughterhouse ovaries were collected from mature sheep, placed in saline (38°C) and transported to the laboratory within 6~8 h. After three washes in fresh PBS medium, ovaries were sliced using a microblade and the contents released in sterile Petri dishes containing fresh PBS supplement 5% (v/v) FBS; 0.054 g L^{-1} heparin sodium salt. Cumulus-Oocyte Complexes (COCs) with several intact cumulus cell layers ranging between 5 and 20 granulosa cell layers and a homogeneous cytoplasm were chosen for maturation *in vitro*. After several washes in OM medium, COCs were cultured in 35 mm Petri dishes (50 oocytes per dish) containing 1 mL of OM medium at 38.5°C in a humidified atmosphere of 5% CO_2 and 95% air. After 20~22 h of maturation, the cumulus cells were removed from the matured oocytes by vortexing the COCs for 3~5 min in Ca^{2+}-Mg^{2+} free PBS medium containing 0.2% (w/v) hyaluronidase. Denuded oocytes were selected for presence of a polar body in PBS medium supplement 5% (v/v) FBS under an inverted microscope (Nikon) and preparation for enucleated.

Oocyte enucleation and pre-damaged mitochondria by photosensibilization: Matured sheep oocytes placed in a 50 μL handling medium drops overlaid with mineral oil at 38.5°C for 20 min then mounted on micromanipulators (NT-88 NE, Narishige, Nikon) equipped with epifluorescence. Each oocyte was held with a holding micropipette (20~30 μm inner diameter, 100~150 μm outer diameter), the first polar body and adjacent cytoplasm containing the metaphase-II chromosomes were removed using an aspiration pipette (15~18 μm inner diameter, 20~25 μm outer diameter) (Fig. 1 a-c). The removed cytoplasm was checked for the presence of chromosomes and polar body by exposure to UV light. Enucleated oocytes mitochondria were damaged by photosensibilization using the mitochondrion-specific fluorophore ROD-123 as described earlier (Thouas *et al*., 2004). Oocytes were placed in mitochongdrial stain medium for 10 min in 35 mm Petri dishes at 38.5°C in a humidified atmosphere of 5% CO_2 and 95% air.

Loaded oocytes were rinsed twice in PBS medium supplement with 5% (v/v) FBS then stored in drops of the handling medium under micromanipulators and irradiated with focused visible light generated from a 100 W mercury bulb and filtered through a dichroic mirror (480±10 nm) to excite ROD-123. Different test groups oocytes were irradiated for periods of 0, 20, 40 or 60 sec, separately before nuclear transfer (Fig. 2a and b). Different control groups oocytes were not loaded ROD-123 and irradiated for the same periods as different test groups.

Goat fetal fibroblasts preparation and pre-damaged mitochondria by photosensibilization: Goat fetal fibroblasts cells were isolated from a day 30-33 goat fetuses. After washed several times in D-Hanks medium and removal of the head and internal organs, the

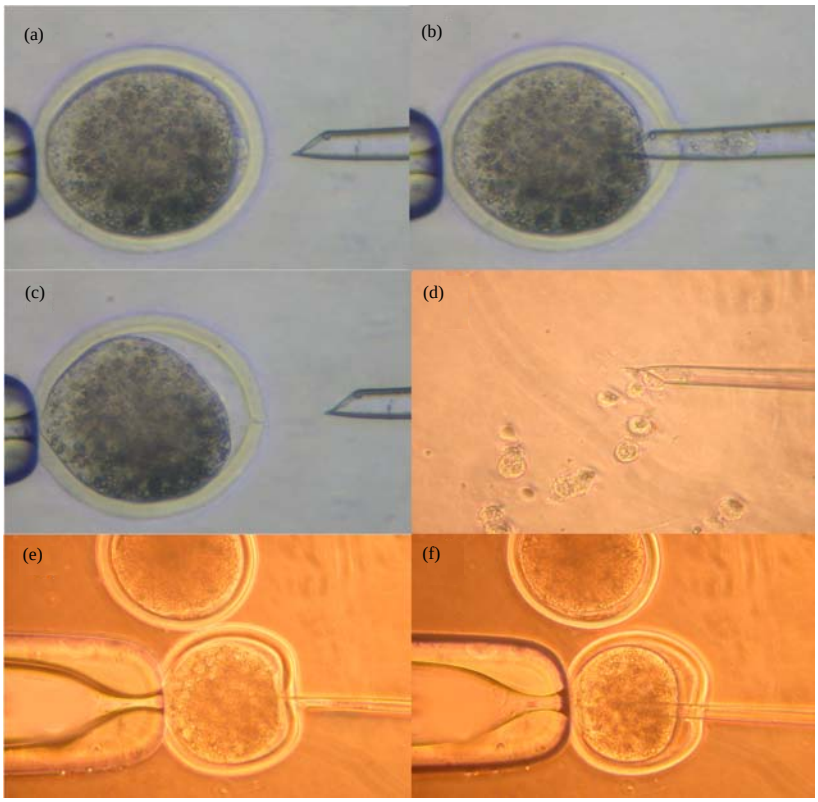

Fig. 1: The procedure of somatic nuclear transfer. a) Before enucleated; b) Enucleation; c) After enucleated; d) Aspirated donor cell; e) Before microinjecting donor cell and f) Microinjecting donor cell

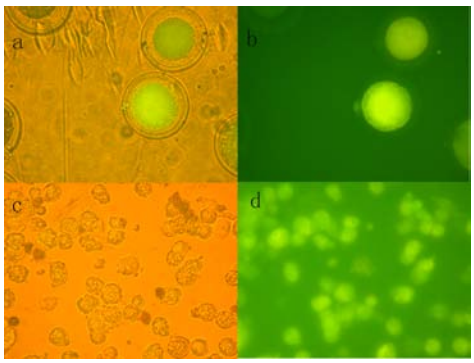

Fig. 2: Enucleated oocytes and donor cell pre-damaged mitochondria by photosensibilization after loaded ROD-123. a) enucleated oocytes in visible light and fluorescence; b) enucleated oocytes in fluorescence; c) donor cell in visible light and fluorescence; d) donor cell in fluorescen

remaining tissues were mechanically dissociated. Explants were placed in 60 mm sterile plastic culture dishes at 37°C in a humidified atmosphere of 5% CO_2 and 95% air for 2~3 h. When explants were damp-dry and adhered to the bottom of plastic culture dishes, DMEM supplement with 5% (v/v) FBS, 100 IU mL^{-1} penicillin and 100 μg mL^{-1} streptomycin were added to each culture dishes and explants were cultured at 37°C in 5% CO_2 and 95% air. While the explant cultures contained a mixed population of cells, fetal fibroblasts were predominant. When the cells from the explants reached 70% confluency, treated with D-Hanks medium supplemented with 0.25% (w/v) trypsin-0.02% (w/v) EDTA at 37°C for 2~3 min. When fetal fibroblasts was off from the bottom of culture dishes but epithelioid cells were still adhered, the same volume of DMEM supplemented with 10% (v/v) FBS was added in for stopping digestion. Cells were washed twice in D-Hanks medium by centrifugation at 1000 rpm for 5 min and seeded into three 60 mm plastic culture dishes. After 2~3 sub-passages, cultured cells were starved with DMEM supplement with 0.5% (v/v) FBS for 2~3 days inducing cells into G_0 phase.

Before nuclear transfer, starved fetal fibroblasts were placed in mitochongdrial stain medium for 10 min then

Table 1: The effect of photosensitization time on early development ability of goat-sheep interspecies somatic nuclear transfer embryos

Time of photo-sensitization (sec)	Sheep oocytes	Goat donor cells	No. of nuclear transfer embryos	Rate of 2-cells embryos (%)	Rate of 8-cells embryos (%)	Rate of morulae and blastocyst embryos (%)
0	Control	Control	96	52.1 (50/96)[a]	62.0 (31/50)[a]	16.1 (5/31)[a]
	(ROD-123)	Control	98	50.0 (49/98)[a]	53.1 (26/49)[a]	15.4 (4/26)[a]
	Control	(ROD-123)	81	53.1 (43/81)[a]	58.1 (25/43)[a]	16.0 (4/25)[a]
	(ROD-123)	(ROD-123)	78	48.7 (38/78)[a]	55.3 (21/38)[a]	14.3 (3/21)[a]
20	Control	Control	92	46.7 (43/92)[a]	58.1 (25/43)[a]	16.0 (4/25)[a]
	(ROD-123)	Control	86	34.9 (30/86)[b]	56.7 (17/30)[a]	5.9 (1/17)[b]
	Control	(ROD-123)	76	51.3 (39/76)[a]	61.5 (24/39)[a]	16.7 (4/24)[a]
	(ROD-123)	(ROD-123)	88	36.4 (32/88)[b]	50.0 (16/32)[a]	6.3 (1/16)[b]
40	Control	Control	87	50.6 (44/87)[a]	61.4 (27/44)[a]	14.8 (4/27)[a]
	(ROD-123)	Control	95	25.3 (24/95)[c]	58.3 (14/24)[a]	7.1 (1/14)[b]
	Control	(ROD-123)	83	48.2 (40/83)[a]	60.0 (24/40)[a]	16.7 (4/24)[a]
	(ROD-123)	(ROD-123)	98	26.5 (26/98)[c]	57.7 (15/26)[a]	6.7 (1/15)[b]
60	Control	Control	92	51.1 (47/92)[a]	57.4 (27/47)[a]	14.8 (4/27)[a]
	(ROD-123)	Control	88	12.5 (11/88)[d]	54.5 (6/11)[a]	0 (0/6)[b]
	Control	(ROD-123)	73	47.9 (35/73)[a]	48.6 (17/35)[a]	17.6 (3/17)[a]
	(ROD-123)	(ROD-123)	74	10.8 (8/74)[d]	50.0 (4/8)[a]	0 (0/4)[b]

The different small letters in the same column indicate significance at 0.05 level. ROD-123 indicated oocytes or donor cells dyed with ROD-123; control indicated oocytes or donor cells did not dyed with ROD-123

washed twice in PBS. Loaded cells stored in a drops of PBS medium supplement with 5% (v/v) FBS, 3% (w/v) PVP and irradiated for periods of 0, 20, 40 or 60 sec, separately as described above. Control cells were not loaded ROD-123 and irradiated separately for the same periods as loaded cells (Fig. 2c and d).

Microinjection, fusion, activation and embryo culture: Fetal fibroblasts were gently aspirated in and out of a injection micropipette (10~15 μm inner diameter, 15~25 μm outer diameter) in a drops of PBS medium supplement with 5% (v/v) FBS, 3% (w/v) PVP until their membrane were broken (Fig. 1d). Then, each cell was injected into a separated encleated oocyte ooplasm (Fig. 1e and f). Reconstructed embryos transferred into SOFaa medium and held there for 2 h at 38.5°C in a humidified atmosphere of 5% CO_2 and 95% air then activated for 5 min in SOFaa medium containing 5 μmol L^{-1} ionomycin. Activated embryos were immediately transferred into SOFaa medium containing 5 mmol L^{-1} 6-DMAP and cultured for 4 h. Then, all reconstructed embryos were washed twice and cultured in SOFaa medium overlaid with mineral oil at 38.5°C in a humidified atmosphere of 5% CO_2 and 95% air. SOFaa medium was half-refresh per 24 h. At the different time of 24~36 h; 3~4 and 5~7 days after reconstructed embryos cultured, 2 cells stage; 8 cells stage and morulae/blastocyst stage embryos were observed and calculated percent separately.

Data analysis: Different treated and control groups repeated 3 times. For the different comparisons involving reconstructed embryos 2 cells; 8 cells and morulae/blastocyst stage development rate of ROD-123 treated and control groups, Chi-squared analysis was used to assess significance levels and considered at 5% probability. Statistical comparisons are reported in the Table 1.

RESULTS

The effect of sheep oocyte mitochondrial pre-damaged on goat-sheep interspecies somatic nuclear transfer embryo early development ability: From 1st and 2nd line of different photoirradiating time in Table 1, researchers can see that the 2 cells rate of ROD-123 treated groups reconstructed embryos declined corresponding to prolong photoirradiating time and difference is significant ($p>0.05$) while the controlled groups reconstructed embryos 2 cells rate did not changed according to photoirradiating time. In the period of 2-8 cells, both treated groups and controlled groups reconstructed embryos development ability did not changed according to photoirradiating time. But in the period of 8 cells to morulae/blastocyst, treated groups reconstructed embryos development ability again declined contrasted with controlled groups and the difference is significant ($p>0.05$). So, the sheep ooplasmic mitochondria play a important role in the event of goat-sheep interspecies nuclear transfer embryos cleavaged. Once nuclear transfer embryos cleavaged, ooplasmic mitochondria can not affect their development ability from 2-8 cells period. But sheep ooplasmic mitochondria can affect goat-sheep interspecies nuclear transfer embryos development ability from 8 cells to blastocyst period and result nuclear transfer embryos development arrest after 8 cells period.

The effect of goat nuclear donor cell mitochondrial pre-damaged on goat-sheep interspecies somatic nuclear transfer embryo early development ability: From 1st and

3rd line of different photoirradiating time in Table 1, researchers can see that lengthening nuclear donor cell photoirradiating time does not affect both ROD-123 treated groups and controlled groups goat-sheep interspecies reconstructed embryos development ability before morulae/blastocyst period. Contrasted with controlled groups, treated groups reconstructed embryos morulae/blastocyst rate increased slightly but the difference is not significant ($p<0.05$).

The effect of both nuclear donor and ooplasmic recipient mitochondrial pre-damaged on goat-sheep interspecies somatic nuclear transfer embryo early development: From 2nd and 4th line of different photoirradiating time in Table 1, researchers can see that both nuclear donor and ooplasmic recipient mitochondrial pre-damaged, goat-sheep interspecies reconstructed embryos development pattern was similar to that of only nuclear donor mitochondria pre-damaged. Lengthening photoirradiating time of ROD-123 treated groups both nuclear donor and ooplasmic recipient can decline reconstructed embryos 2 cells rate and 8 cells to blastocyst period development ability but did not affect 2-8 cells period development ability.

DISCUSSION

Mitochondria is the producer of ATP in eukaryotes. ATP can be produced through the Kreb' cycle, β-oxidation and Oxidative Phosphorylation (OXPHOS). In the vast majority of case, the primary pathway for ATP production is OXPHOS via the electron transfer chain which unlike any other cellular pathway is encoded by two distinct genomes, the nuclear and mitochondrial genomes (Justin *et al*., 2004). Mammalian mtDNA is double chain circular DNA, approximately 16.5 kilobases, encodes 13 subunits of respiratory chain enzymes and 22 tRNAs and 2 rRNAs of the mitochondrial translation apparatus but several hundred other mitochondrial components are encoded in the nucleus, requiring extensive nuclear-mitochondrial interactions for proper mitochondrial function (Garesse and Vallejo, 2001). Oocyte mitochondria are morphologically primitive or immature such as reduced organelle size and less complex internal structure compared with those of somatic cells (Thompson *et al*., 2002; Trimarchi *et al*., 2000). In spite of this immaturity, metabolic evidence suggests that the mitochondria of the oocyte and early embryo are constitutively active and maintenance of this low-level activity is necessary for ongoing development (Thouas *et al*., 2004). In this research, the sheep oocyte mitochondrial damage can result in goat-sheep interspecies somatic nuclear transfer reconstructed embryos 2 cells rate declined. The probably reason of this is that some amount of ATP supply is essential for nuclear transfer reconstructed embryos starting the cleavage after nuclear donor cell finished reprogramming. But the oocyte mitochondrial damage can not affect the developmental ability of embryos reconstructed in 2-8 cells stage. It probably due to the reconstructed embryos need small amount of energy during this development period if small amount of not damaged mitochondria can support reconstructed embryos cleavage, it also can support reconstructed embryos 2-8 cells period development. In the process of embryo development, the Zygote Gene Activation (ZGA) is important event in the period of transition from maternal hereditary factor to zygote gene regulation. In the process of maturation, mammal oocytes had synthesized and accumulated large amount of protein, mRNA, organelle which constituted the maternal hereditary factor for supporting embryo early development. After oocyte maturation and fertilized, these maternal hereditary factor controlled embryo early development until ZGA. Then embryo further development was controlled by mRNA and protein coded by nuclear gene. The time of ZGA occurred was different according to different kind of animals. The time of ZGA occurred in goat was in 2 or 8 cells stage (Chartrain *et al*., 1987; Pivko *et al*., 1995) and in sheep was 8-16 cell stage (Crosby *et al*., 1988).

Sheep oocyte mitochondrial damage resulting in goat-sheep interspecies somatic nuclear transfer reconstructed embryos arrest after 8 cells period maybe due to embryo development control transition from maternal hereditary factor to zygote gene. After 8 cells period, goat somatic nuclear gene was activated, transcription and translation cellular factors and protein subunits which can control sheep mitochondrial replication and OXPHOS pathway but goat mitochondria were destroyed by an unclear pathway or diluted for can not be replicated. After some amount of sheep mitochondria was damaged, the number of mitochondria was declined in interspecies somatic nuclear transfer reconstructed embryos due to lack of sufficient mitochondrial replication templates and the same time reconstructed embryos development need more energy after 8 cells stage, thus causes reconstructed embryos can not obtain sufficient energy for it further development and arrested.

Nuclear transfer can result in donor cell mitochondria entered into recipient ooplasm. So, the mitochondria derived from donor cell and recipient ooplasm can coexist in nuclear transfer reconstructed embryos. Thus, could possibly cause the nuclear transfer embryos, even the

offspring, display four kinds of mitochondria hereditary types as following: the first kind of possible type is that mitochondria derived from nuclear donor cell was destroyed by proteolysis in oocyte lysosome and proteasome, the mitochondria in nuclear transfer reconstructed embryos and offspring were all derived from ooplasmic recipient (Evans *et al*., 1999). Second, mitochondria derived from ooplasmic recipient was destroyed, nuclear transfer reconstructed embryos and offspring mitochondrial were all derived from nuclear donor cell (Chen *et al*., 2002). Third, mitochondria derived from both nuclear donor cell and ooplasmic recipient coexist in all kinds of tissue and cells of offspring (Takeda *et al*., 2003). Forth, mitochondria was heteroplasmic in offspring certain tissue and cells and homoplasmic in other tissue and cells (Hiendleder *et al*., 2003). In this research, nuclear donor cell mitochondrial damage can not affect goat-sheep interspecies somatic nuclear transfer reconstructed embryos development ability before morulae/blastocyst. It implied that mitochondria derived from nuclear donor cell was damaged through certain pathway or can not be replicated and diluted gradually in the process of embryo development, recipient ooplasmic mitochondrial occupied the dominant position in the end.

In this research, both nuclear donor and ooplasmic recipient mitochondrial damaged, the goat-sheep interspecies somatic nuclear transfer reconstructed embryos development pattern was similar to that of only nuclear donor mitochondrial damaged which further proved that ooplasmic recipient mitochondria is more important than nuclear donor mitochondria in the period of goat-sheep interspecies somatic nuclear transfer embryo early development.

CONCLUSION

The result of this research showed that oocyte mitochondria play an important role in the period of interspecies somatic nuclear transfer embryo early development which indicated the oocyte quality have the important influence on interspecies somatic nuclear transfer efficiency. So, further enhancing quality of matured oocyte and reduced damage on oocyte in the process of nuclear transfer would benefit to interspecies somatic nuclear transfer efficiency.

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