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Development of nuclear transfer goat embryos derived from *in vitro* matured oocytes as recipient cytoplasm and blastomeres, fetal fibroblasts and cumulus cells as nuclear donors

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ABSTRACT

In vitro development of goat nuclear transfer embryos produced using *in vitro* matured oocytes as recipient cytoplast and blastomeres, fetal fibroblast and cumulus cells as nuclear donors, was investigated. The constructed oocytes were fused and activated by electrical and chemical activation respectively. Activated nuclear transferred embryos (NTEs) were incubated overnight in tissue culture medium 199 (TCM199) +15% fetal bovine serum (FBS) at 38.5°C in CO_2 incubator and cultured subsequently on monolayer of goat oviductal epithelial cells in TCM199+15% FBS. The fusion rates for blastomere, fibroblast and cumulus cells were 76.1, 55.7 and 57.7% respectively. The proportion of the NTEs cleaved to 2-cell were 42.2, 44.3 and 40.0% for blastomere, fibroblast and cumulus cells; respectively; 27.9, 16.3 and 12.5% of the cleaved embryos derived from blastomere, fibroblast and cumulus cell developed to blastocyst *in vitro.* It is concluded that the efficiency of *in vitro* development of goat NTEs produced from *in vitro* matured oocytes and blastomeres is better. However none of the recipient delivered offspring following transfer of nuclear transfer goat embryos into recipient goats.

Key words: Blastomeres, Goat, *In vitro* matured oocytes, Nuclear transfer, Somatic cells

Live offspring have been obtained following nuclear transfer (NT) of embryonic, fetal, and adult cells in various laboratory and domestic animals (Keefer 2008). However, the overall efficiency of the NT for production of the live offspring still remains low (Cibelli 2007). Initially *in vivo*-matured oocytes were used as recipient cytoplasm for NT of blastomeres in cattle (Prather *et al.*1987) and sheep (Willadsen *et al.* 1986). However, due to the high cost of *in vivo* produced oocytes, *in vitro*-matured (IVM) oocytes are used now (Barnes *et al.* 1993, Keefer *et al.*1993), although lower production of lambs from NT embryos derived from *in vitro*-matured oocytes than from ovulated oocytes was reported (Wells *et al.* 1997). Most of the nuclear transfer studies in goat were conducted with ovulated (Baguisi *et al.* 1999, Behboodi *et al*. 2004, Park *et al.* 2006, Yuan *et al.* 2009, Zou *et al.* 2001, 2002) or oocytes collected from super ovulated animals by laparoscopic ovum pick-up (OPU-derived) (Keefer *et al.* 2000, 2001, 2002, Melican *et al.* 2005, Ohkoshi *et al.* 2003). Successful production of kids involving the use of *in vitro* matured oocytes and somatic cells as nuclear donors was reported in a few instances (Chen *et al.* 2007, Lan *et al*. 2006, Reggio *et al.*2001). However, the efficiency of development of goat nuclear transfer embryos (NTE) produced from *in vitro* matured oocytes and blastomeres and somatic cells as nuclear donors was never directly compared. Therefore the present study compared the development of goat NTE produced by transferring blastomeres, cultured fetal skin fibroblasts or contemporary cumulus cells into IVM oocytes.

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MATERIALS AND METHODS

Superovulation of embryo donor goat does: Regularly cycling goat does were selected for superovulation. Superovulation protocol as described by Rao *et al.* (1984) was used. Each female goat was fed with melengestrol acetate@ 1.15/day/animal, for 16 days. On day 16 of MGA feeding the donor females were administered subcutaneously with 500 IU of pregnant mare serum gonadotropine to induce superovulation. Starting from 48 h after pregnant mare serum gonadotropine administration heat detection was carried out every 4 h round the clock. As soon as a doe was detected in estrus she was given i/v injection of 750 IU of human chorionic gonadotropin. Each superovulated doe was also

allowed to mate with 2 fertile bucks every 12 h from the beginning of estrus till she refused to accept the male.

Collection of goat embryos: Between 96 to 120 h after the onset of estrus, each embryo donor doe was subjected to laparotomy and the genital tract was exposed. Each oviduct was flushed from utero-tubal junction towards fimbriated end with 10–15 ml of PBS supplemented with 10% FBS and antibiotics (penicillin 100 U/ml and streptomycin 100 µg/ml). The collected medium was allowed to settle for 30 min and then morula stage embryos were isolated under a stereo zoom microscope.

Oocyte isolation and maturation: All the procedures for *in vitro* maturation were carried out according to Rao *et al.* (2002). Briefly goat ovaries were aseptically collected from does of mixed breeds from Chennai slaughterhouse, a distance of about ~200 km and transported to the laboratory within 5–6 h of slaughter, in prewarmed phosphate buffered saline (30–35°C) supplemented with penicillin (200IU/ml) and streptomycin (200µg/ml) PBS. Oocytes from apparently non-atretic follicles (≥3 mm) were aspirated into a 5 ml disposable syringe containing HEPES buffered tissue culture medium 199 (TCM199H) +10% FBS+ 50 µg/ml gentamicin + 25 IU/ml heparin. Morphologically excellent to good quality cumulus-oocyte complexes (COC) having 3–5 layers of cumulus cells (Fig. 1b) and evenly granulated cytoplasm were washed several times in oocyte handling medium (TCM199H +10% FBS+ 50 µg/ml gentamicin), followed by 3 washes in maturation medium (bicarbonate buffered TCM199 (TCM199B) was supplemented with 10 µg/ml follicle stimulating hormone,10 µg/ml luteinizing hormone, 1µg/ml estradiol-17ß,50 µg/ml gentamicin sulphate,10µg/ml bovine serum albumin, and 20% (v/v) estrus goat serum). COCs were cultured in the maturation medium for 27 h on monolayers of goat granulosa cells at 38.5°C, in 5% CO_2 in air, in a humidified (95%) incubator.

Preparation of fetal fibroblasts: For the culture of fibroblast cells, tissues biopsies were collected aseptically from the skin of 5 months old goat fetus obtained from slaughterhouse. The tissue was cut into small pieces and incubated in 0.25% (w/v) trypsin at 37°C for 30 min. The trypsinized pieces were seeded in 60 mm cell culture dishes with Dulbecco's modified eagles medium (DMEM) supplemented with penicillin (100 IU/ml), streptomycin (100 µg/ml) and 20% (v/v) FBS. After 2 to 3 days, a fibroblast cell layer attached to the flask bottom.The culture medium was changed every 2 days. Fibroblast cells were serum starved at 70–80% confluence (Fig.1a) for 3 days and detached by 0.25% trypsin and 1 mM EDTA prior to NT.

Preparation of donor cells for nuclear transfer

Blastomeres for nuclear transfer: Morphologically normal and compact morulae with uniform sized blastomeres were considered suitable for nuclear transfer. One or two selected embryos were incubated in 0.1% pronase solution (37°C) for ~2 min while observing continuously for digestion of zona under stereo-zoom microscope. Blastomere clusters were separated from the embryos by application of gentle sucking force with a pipette having smaller diameter than the embryo. Blastomere clusters were incubated in 20 µl droplet of Hanks balanced salt solution without calcium and magnesium (HBSS) for 20 min. Finally each blastomere mass was transferred to a 20 µl droplet of collection medium. Blastomeres were separated by repeated pipetting with a glass pipette of 20–25 µm inner diameter. The blastomeres were kept at 8–10°C till used for nuclear transfer.

Fetal fibroblast cells: Serum starved cells were washed 3 times with PBS and harvested from the culture plates by incubating with trypsin-HBSS solution for 5 min. The separated round cells were washed 3–4 times by centrifugation (200 rpm/5 min) and 100–200 cells were transferred into the micromanipulation chamber.

Cumulus cells: Twenty-seven hours after *in vitro* maturation, oocytes with cumulus cells were placed in 20 µl droplet of HBSS under mineral oil and kept in an incubator for 20 min. After the incubation individual cumulus cells were separated by repeated pipetting through a fine bore glass pipette. The separated cells were placed in 20 µl droplet of collection medium covered with mineral oil in a 35 mm culture dish. The dish was kept at room temperature till used.

Enucleation: After *in vitro* maturation, those oocytes with extruded first polar body and uniform cytoplasmic granularity (Fig.1c) were selected for nuclear transfer. Enucleation and NT procedures were carried out according to Rao *et al*. (1998). All micromanipulations were carried out at room temperature on a microscope equipped with epifluorescence. All micromanipulation tools were prepared in our laboratory using micropipette puller, micro beveller and microforge. Oocytes were stained with Hoechst 33342 (10 µg/ml) for 20 min prior to enucleation. Briefly, a group of 5 oocytes were placed into a micromanipulation chamber containing handling medium supplemented with cytochalsin-B (5.0 µg/ml) and secured with a holding pipette (Fig.1d). The enucleation pipette was penetrated into the perivitelline space and the polar body and a small amount of surrounding cytoplasm consisting of M-II spindle was aspirated into enucleation pipette (Fig.1e). Enucleation was confirmed by a brief exposure of the contents in the pipette to UV light (Fig.1e).

Nuclear transfer: Smooth surfaced and uniform and medium sized blastomeres, fetal fibroblast and cumulus cells were selected for NT. A single donor cell was placed into the perivitelline space of the enucleated oocyte using the enucleation pipette (internal diameter, 20–25 µm for blastomere and fibroblast cells and, 10–15 µm for cumulus cells) through the same slit in the zona made during enucleation.

Electrofusion and activation: After nuclear transfer, donor cell-oocyte pairs were washed 2 times in collection medium and fusion mediun (0.3 M mannitol, 100 µM $CaCl_2$ and 50

μM $MgCl_2$) and finally transferred into a 0.5 mm gap electrode fusion chamber containing fusion medium. Cell fusion was induced with single DC electric pulses of 1KV/cm × 60μs (blastomeres), 1.3 KV/cm × 25μs (fibroblast cells) and 1.3.KV/cm × 15μs (cumulus cells) delivered by an electro cell manipulator. These fusion parameters were standardized earlier (data not shown) to suit different donor cells. The fused couplets were activated by subjects to similar set of fusion pulse. Immediately after activation pulse the couplets were washed thoroughly with handling medium. Subsequently, fused couplets were activated by 5 min exposure to 5 μM ionomycin and washed thoroughly in culture medium. These incubated for 3 h in 2 mM of 6-DMAP prepared in culture medium. Following activation the couplets were washed in cultured medium.

Oviducal cell culture: Oviducts obtained at the local slaughterhouse were transported to the laboratory in PBS plus penicillin (200 IU/ml) and streptomycin (200 μg/ml) at 35–38°C. Each oviduct was straightened by trimming free of connective tissue and attached fat and was washed thrice in PBS. Both fimbriated end and uterine end were clamped with haemostatic forceps and then immersed in 70% ethanol bath for 30–40 sec, taking care to avoid entry of alcohol into oviducts. Then they were rinsed in PBS with antibiotics. Each oviduct was filled with HBSS from uterine end. Oviducts with HBSS were kept in an incubator (38.5°C) for 20 min. Each oviduct was cut at the clamped fimbriated end and the fluid was allowed to drain into a 15 ml centrifuge tube. The oviduct lumen was opened from frimbriated end to uterine end and mucous was scrapped with a surgical blade (No.22) and added to the same test tube along with additional 2–4 ml of HBSS. Scraped cell clumps were pipetted several times through a 1 ml micropipette to break up the clumps of the cells into individual cells. These tubes containing oviducal epithelial cells were centrifuged at 2,000 rpm for 5 min. Supernatant was discarded and the pellet was re-suspended in 1–2 ml of cell culture medium. About 250 μl of this suspension containing 2×10^6 cells/ml was distributed in each of the 4 wells of a culture plate; 750 μl of culture medium was added to this cell suspension in each well. The cells were cultured for 5–6 days in a carbon dioxide incubator at 38.5°C to establish monolayer (Fig.1f). Every 48 h, 500–600 μl of culture medium in each well was replaced with fresh culture medium.

In vitro *culture of NTEs:* Fused couplets were cultured on monolayer of ovidutcal epithelial cells in TCM199B supplemented with 15% FBS in a humidified atmosphere of 5% CO_2 in air at 38.5°C. Embryos were evaluated every 24 h for development (Fig.1 g - k).

Embryo transfer of NTEs: The recipient does were selected from those exhibiting natural estrus 1–3 days prior to scheduled embryo transfer so that the NTE were transferred to recipient 24–60 h after initiation of IVM. Recipient does were fasted for 24h prior to surgery. The ventral surface of the abdomen was shaved off hair and cleaned with antiseptic solution. The does were anesthetized by injection of xylazine (0.2 mg/kg body weight) and 1 ml of ketamine (4.4 mg/kg bodyweight) intramuscularly. The reproductive tract was exteriorized through a mid ventral incision to allow visual conformation of CL on the ovaries gently pulled out and held outside of the abdominal cavity by forceps. Transferable nuclear transfer embryos (3–6) in a small volume (~ 5–7 μl) of culture medium were loaded into glass pipettes. The pipette was introduced into the oviducts through the ostium abdominale up to 1 cm depth and the embryos were gently released by pushing the plunger of the syringe. Transfer of additional 3–6 embryos was undertaken into the other oviduct. External surface of fimbriae and oviducts were washed with PBS. Operated animals were monitored for post operative care for 7 days. The animals were observed for signs of pregnancy or return to estrus.

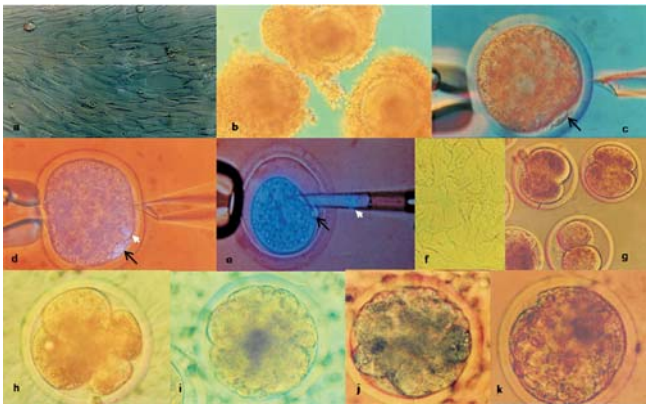

Fig. 1. Photographic representation of *In vitro* development of nuclear transfer embryos in goat using *in vitro* matured oocytes as recipient cytoplasm and fetal fibroblasts as nuclear donors. **a.** Goat fetal skin fibroblast cell monolayer, **b.** goat oocyte before *in vitro* maturation: 3–5 layers of cumulus cells, **c.** *in vitro* matured goat oocyte (arrow indicates polar body), **d.** position of polar body (black arrow) and MII (white arrow) for enucleation (HOECHST stained, **e.** enucleation in progress (polar body (black arrow) and MII (white arrow) in enucleation pipette, **f.** goat oviductal cell monolayer, nuclear transfer embryos, **g.** 2-cell, **h.** 2-cell, **i.** 4-cell, **j.** 16-cell, **k.** morula, **l.** blastocyst.

RESULTS AND DISCUSSION

A total of 680 cloned goat embryos were produced of which, 402 were from blastomeres, 174 from fetal fibroblasts and 104 from contemporary cumulus cells (Table 1).

Among the cloned goat embryos produced using blastomeres, cultured fetal fibroblasts and cumulus cells, oocytes constructed with blastomeres gave significantly higher fusion rates than other nucleus donor cells (Table 1). This may be related to the fact that blastomeres are relatively bigger and therefore easy to orient properly during fusion. All the publications on blastomere cloning in goat were undertaken with ovulated oocytes rather than *in vitro* matured

Table 1. *In vitro* development of nuclear transfer goat embryos

Type of nucleus	No. of NTE (replicates)	No. fused (%)	No. cleaved/ no. cultured (%)	No. of blastocyst/ no. cleaved (%)
Blastomere	402 (24)	306/402 (76.1)[a]	129/306 (42.2) [a]	36/129 (27.9) [a]
Fetal fibroblast	174 (14)	97/174 (55.7)[b]	43/97 (44.3) [a]	7/43 (16.3) [b]
Contemporary cumulus cell	104 (12)	60/104 (57.7)[b]	24/60 (40.0)[a]	3/24 (12.5)[b]
Total	680 (50)	463/680 (68.1)	196/463 (42.3)	46/196 (23.5)

Values with same superscript within a column are not significantly different; normal deviate test for proportions; P<0.5.

ones (Behboodi *et al*. 2004, Yong *et al.* 1991, Yong and Yuqiang 1998). Thus results obtained in our study cannot be compared to earlier reports. The rate of first cleavage in different groups of cloned goat embryos was similar (Table 1). As chemical activation of all the NTE whether blastomere or somatic cell cloned was undertaken, the rate of cleavage could be reasonably expected to be similar. However, development to blastocyst stage was significantly better in blastomere-cloned group than the other 2 groups (Table 1). This indicated that blastomere nucleus undergoes reprogramming relatively easily. Further, blastocyst formation rates obtained in this study with blastomere cloned embryos are lower than those reported by Yong and Yuqiang (1998). This may be ascribed to the *in vitro* matured oocytes from slaughter house ovaries employed in the present study. Further in the present study oocytes were harvested from slaughter house ovaries irrespective of the age and reproductive status of the animal which may have had an adverse influence on the development potential of NTE (Betthauser *et al.* 2000). *In vitro* development of SCNT embryos to blastocyst obtained in this study was higher than that from other reports (Chen *et al*. 2007, Das *et al*. 2003, Lan *et al*. 2006, Zhang *et al.* 2004,Akshey *et al.* 2010) and similar to that of Zhang *et al*. (2008) and lower than that of the results (cleavage and blastocyst formation rate of 67.41% ± 3.92 and 26.96% ± 3.86) obtained with and cultured cumulus cells (Akshey *et al*. 2010) and goat adult fibroblasts (Dutta *et al.* 2011) fused with zonafree goat oocytes using handmade cloning (Dutta *et al*. 2011).

On transfer of 2-cell embryos (134), to recipient goats, none of the blastomere or somatic cell cloned embryos developed to live offspring in this study. This is surprising since the *in vitro* development was relatively better. In earlier reports offsprings were produced from blastomere cloned embryos (Yong *et al*. 1991, Yong and Yquiang 1998). Similarly fibroblast cell cloned (Baguisi *et al*. 1999, Behboodi *et al.* 2004, Keefer *et al.* 2001, 2002, Melican *et al.* 2005, Zou *et al.* 2002), cumulus cell cloned embryos (Cheng *et al.* 2002, Keefer *et al*. 2000, Zou *et al*. 2001) also developed to live young. However, in all the reports on development of blastomere and somatic cell cloned embryos to offspring, ovulated (*in viv*o matured) or OPU derived oocytes were used, whereas in the present study only *in vitro* matured oocytes were used. The present study is the first attempt at producing cloned goats using blastomeres and *in vitro* matured oocytes from abattoir source. Thus it is likely that the *in vitro* matured oocytes did not achieve the required cytoplasmic maturation to support complete reprogramming of the transplanted blastomere nucleus. However, live kids were successfully produced using *in vitro* matured oocytes from abattoir derived oocytes (Chen *et al.* 2007, Lan *et al*. 2006, Reggio *et al.* 2001). Chen *et al.* (2007) enucleated oocytes at telophase-II rather than M-II and the donor nucleus was directly injected into the oocyte cytoplasm rather than fused to the enucleated oocyte. Thus the NT technique used in this study may not have supported *in vivo* development of NTE. It is concluded that when IVM oocytes were used for NT in the goat, blastomeres rather than somatic cells supported better *in vitro* development.

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