

Reconstruction of *in vitro* matured goat oocytes by using synchronized skin fibroblast cells as donor nuclei

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Cloning of embryos by nuclear transplantation has been developed in several species using foetal fibroblasts as donor cells. In the present study, the developmental potential of foetal fibroblasts, arrested in metaphase stage using cytochalasin-B, was evaluated using nuclear transfer. Studies were undertaken to find out suitable stage of the cell cycle of goatskin fibroblast cells with enucleated *in vitro* matured goat oocytes and their capability of development of embryo. Skin cells were isolated from fetus as well as from adult skin and cultured to monolayer using RPMI-1640 media with 10% FCS in 5% CO₂ incubator at 38.5° C ± 1° C with 95% humidity. Immature oocytes were matured *in vitro* for 22-24 hrs and enucleated using a Leitz micromanipulator. The cytochalasin-B blocked synchronized cells were used as donor cells for transferring into the perivitelline space of the enucleated oocyte and electrofused with constant AC pulse at 7-10 volts, while DC pulses at 180-400 volts were applied for 5-20 µ sec followed by culturing in CO₂ incubator to observe proper electrofusion and cleavage. The embryos after fusion were activated with cytochalasin-B for 2-4 hrs and then transferred to IVC media. Foetal fibroblast became confluence within 2 days whereas adult fibroblast cells took 7-8 days to become confluence and, by 1-2 hr of cytochalasin-B treatment, around 85% foetal fibroblast cells arrested in the metaphase stage and 4.3% reconstructed embryos reached to 2-cell and morula stage in each category. Thus, 3-5 passaged foetal fibroblast cells synchronized by cytochalasin-B and around 300 volts would be better for electrofusion for reconstruction of goat oocytes.

Keywords: goat, reconstructed embryo, nuclear transfer and fibroblasts

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Introduction

The successful production of offspring derived through nuclear transfer (NT) depends upon a wide variety of factors, including appropriate cell cycle coordination. When the donor cell is fused to the recipient oocyte, which is arrested in the second metaphase in meiosis, the nuclear envelope breaks down and the chromosomes condense until the oocyte is activated¹. This condensation phase causes chromosomal defects in donor cells undergoing DNA synthesis. Donor cells in the G1 phase of the cell cycle (before DNA synthesis) however, condense normally and support a high rate of early development². The donor cell should be compatible with the rapid cell divisions that occur during early embryo development. The cells should also be in G1, either by artificially arresting the cell cycle or by choosing a cell type that has an inherently long G1 phase. Live offsprings have been obtained following

NT of embryonic, foetal and adult cells in cattle, sheep, goats, pigs and mice³⁻¹⁰. The source and the treatment of the donor cell and recipient oocyte are key factors in successful outcome of this process. The main hurdle of the technology is a very small number of cell transformations after fusion of donor cells with enucleated oocytes. On electrofusion and subsequent development to blastocyst is another major constraint for applying the technology to the animal production system. The present study envisages to check the developmental potential of cytochalasin-B blocked foetal skin fibroblasts (FF) cells used as donor cells for NT in enucleated goat oocytes matured *in vitro*.

Materials and Methods

All the chemicals and foetal calf serum used were from Sigma Chemical Co., USA. Chemicals for general use of NSS were from Hi-media and plasticwares from Greiner, Labortechnik, Germany. All the culture media were sterilized by filtration through 0.22 µm filter and stored at 4° C for not more than one month.

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***In vitro* Maturation**

Goat ovaries irrespective of the age of the animal and stage of oestrous cycle were collected aseptically from the local slaughter house and transported to the laboratory within 1 hr of slaughter in lukewarm sterile NSS (30-35°C) supplemented with penicillin-G (200 IU/ml) and streptomycin (200 µg/ml). Oocytes from apparently non-atretic surface follicles (≥ 3 mm) were released into a Petridish containing oocyte collection medium (modified PBS, pH 7.2-7.4, osmolarity 280-290 mOsm) by puncturing with 18-gauge hypodermic needle. Morphologically excellent to good cumulus oocyte complexes (COC) having atleast 3-5 layers of cumulus cells investment and evenly granulated ooplasm were washed several times in oocyte collection medium followed by 3 washes given in maturation medium (TCM-199 with 1µg/ml E2, 0.5 µg/ml FSH, 100 IU/ml LH and 10% FCS, pH 7.2-7.4, and 280 mOsm). Then 10-12 COC were cultured in 50 µl droplet of maturation media covered with sterile mineral oil in 35 mm culture dish at $38.5 \pm 1^\circ\text{C}$, 5% CO_2 in humidified (95%) incubator for 20-22 hrs.

Preparation of Foetal and Adult Skin Fibroblast Cells

The gravid uterus, collected from the local slaughterhouse and transported as such to the laboratory within 1 hr, was washed several times with lukewarm water. Then the fetus taken out from the uterus aseptically and washed with sterile NSS (30-35°C) supplemented with streptomycin and penicillin-G. The skin was removed with the help of forceps and scissors, and washed with DPBS. The skin samples were chopped separately in small pieces with sterile scissors and washed 7-8 times with DPBS and finally the cells were isolated by digesting in DPBS (Ca^{2+} and Mg^{2+} free), supplemented with 0.5%(w/v) trypsin (Sigma) and 0.05%(w/v) collagenase (Sigma) for 30 min and 1½ hrs in foetal and adult skin sample, respectively. The released cells were washed 8-10 times (600 rpm/10 min for each washing) with DPBS and finally with the culture media (RPMI-1640+10% FCS) supplemented with streptomycin, penicillin-G and amphotericin-B. The cells were then plated in 25 cm^2 culture flask with 3 ml of culture media at $38.5 \pm 1^\circ\text{C}$, 5% CO_2 in humidified (95%) incubator.

Synchronization of the Cells in Metaphase Stage

The growing cells were induced to metaphase block (after 3-5 passage) by treating with cytochalasin-B (@ 8 µg/ml) for 1-2 hr before use. On adding cytochalasin-B in the culture media, the cells were

incubated at every 30 min interval, and harvested by scrapping with a sterile plastic scraper. Cells were washed twice by centrifugation (600 rpm/5 min) with the culture media and then incubated in a hypotonic solution (0.075 M KCl) for 10 min. The cell pellet was collected by centrifugation (130 g/10 min). It was slowly mixed with chilled, freshly prepared fixative (methanol:glacial acetic acid, 3:1). It was also mixed with remaining small volume of fixative (0.5 ml) and was dropped at arms length onto wet, cold, grease free slides. Slides stained with 2% giemsa stain were examined under phase contrast microscope for chromosome observation. Total 200 cells were observed for working out of the percentage of cells at metaphase stage each time.

Preparation of Donor Cells and Oocytes for NT

Before use, the cells were washed three times by changing the media and harvested from the culture flask by incubating with trypsin-versene solution for 7-8 min. The released cells were washed 3-4 times by centrifugation (600 rpm/10 min) and 100-200 cells were taken into the enucleation droplets. Cumulus cells from *in vitro* matured oocytes (20-22 hrs) were removed by 0.1% trypsin solution in PBS for 5 min followed by repeated pipetting. The oocytes that were totally free from any cumulus cells and having the first polar body were taken into the enucleation droplets as recipient oocytes.

Enucleation of Caprine Oocytes

All micromanipulations were carried out at room temp using Leitz micromanipulator and inverted microscope (Leitz, Labovet, Germany). The recipient oocytes were placed in a 30-40 µl drop of enucleation medium (TCM-199 with cytochalasin-B @ 8 µg/ml). Cutting the part of the zona pellucida near the first polar body carried out enucleation of oocyte, and then a small volume (10-20%) of cytoplasm surrounding the first polar body was aspirated out through the slit made at enucleation with a cutting needle (outer diam 30 µm, inner diam approx. 10-20 µm). The aspirated cytoplasm was fixed on the clean grease free slide, stained the biopsy material with 2% Giemsa stain and enucleation confirmed by visualizing the chromosome material under the microscope.

Microinjection of Donor Cell, Electrofusion, Activation and Culture of Reconstructed Embryos

Single fibroblast cell was transferred individually into the perivitelline space of the recipient oocyte

through the same hole/slit in the zona pellucida made during enucleation to facilitate close membrane contact for subsequent fusion. The nuclear transferred oocytes were washed thrice in TCM-199 supplemented with 20% FCS. Karyoplast and cytoplasm were fused using electric pulse. Reconstructed embryos were washed in 50 μ l of Zimmermann's fusion media (0.3 M mannitol, 0.1mM CaCl_2 , 0.1 mM MgCl_2 , 0.05% BSA fatty acid free) for equilibration for 5 min before electrofusion. Fusion was performed at room temp, in a chamber with two stainless steel electrodes 500 μ m apart overlaid with fusion medium. The karyoplast and cytoplasm complexes/reconstructed embryos were manually aligned with a fine pasteur pipette, so that the contact surface between the cytoplasm and the donor cell was parallel to the electrodes. Cell fusion was induced with one or two DC pulses (180-400 volts) for different pulse durations (5-20 μ sec), delivered by a BTX Electrocell manipulator 200 (BTX, San Diego, CA, USA).

The reconstructed embryos were washed thoroughly with activation media (TCM-199 with cytochalasin-B @ 8 μ g/ml) and then incubated 2-4 hrs for activation. The reconstructed embryos were taken out from the activation media, washed three times with culture media and transferred to 100 μ l droplets of TCM-199 + 10% FCS for culture in a 5% CO_2 incubator at $38.5^\circ \pm 1^\circ \text{C}$ with 95% humidity for 7-8 days. After 48 hrs the cleavage was checked.

Results and Discussion

Monolayer Culture of Skin Fibroblast Cells

Results of parameters pertaining to the preparation of skin fibroblast cell monolayer (Table 1) and their morphological characteristics before and after culture are shown in Fig. 1.

Cell Isolation, Viability and Morphology

Cells, isolated from the adult skin and foetal skin by enzymatic digestion, were individual and round in shape. Approximate live percentage was about 60-70% and 90-95% after trypan blue dye exclusion method in case of adult and foetal sample, respectively (Table 1).

Cell-Synchronization by Using Cytochalasin-B

To get the synchronized cell stage in metaphase, fibroblast cells were treated with metaphase arrester i.e. cytochalasin B @ 8 μ g/ml. In FF cells, at 0 hr of the treatment 9.5% of cells were in metaphase stage, after $\frac{1}{2}$ hr the arrested population was 64%, but after 1 hr the arrested cell population, as it was found 85% (Table 2). After $1\frac{1}{2}$ to 2 hrs, the number of arrested cells did not increase significantly. When adult fibroblast (AF) monolayer treated in similar way, at 0 hr of the treatment 8% of cells were in metaphase

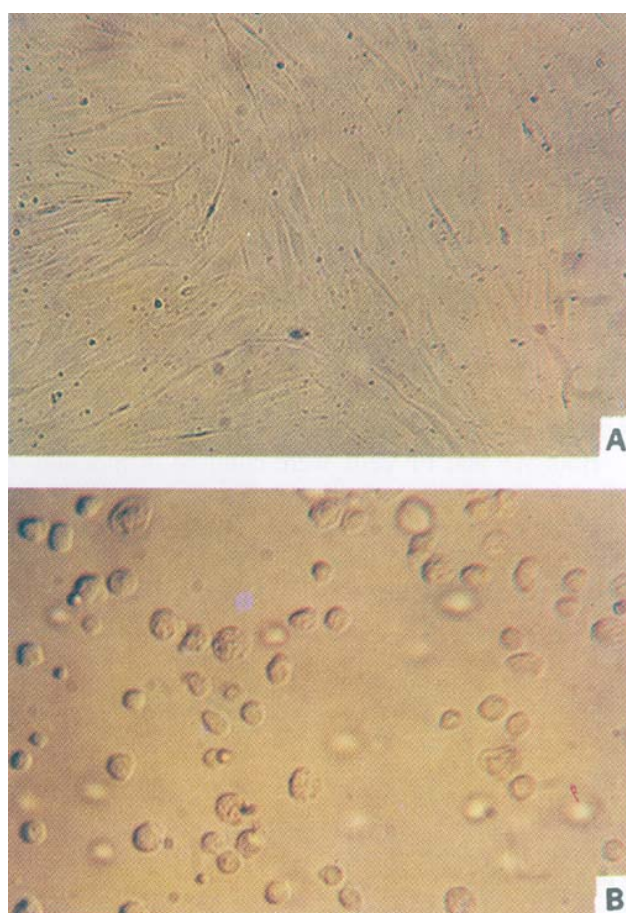


Fig.1—A. *In vitro* cultured caprine foetal skin fibroblast cells monolayer at passage 4 ($\times 400$); B. Fibroblast cells harvested by standard trypsinization from monolayer just before microinjection ($\times 400$).

Table1— Isolation, attachment, confluence and survival of skin fibroblast cells used in the experiment

Cell type	Live (%) (approx.)	Attachment to surface after seeding (hrs)	Time to confluence (Days)	<i>In vitro</i> survival observed (passage)
Adult	60-70	24-36	7-8	4-5
Foetal	90-95	3-6	2-3	10-12

stage, after 2 hrs, the arrested cell population was 9.5%, but after 8 hrs the number increased to 66.5%. Total 200 cells were screened each time for cell stage identification in each experiment.

Development of Reconstructed Embryos Using Donor Cells

FF cells, which have growth performance and synchronization of cell cycle better than the AF cells, were selected as donor cells. Using cytochalasin-B, different developmental stages of reconstructed embryos blocked FF cells as donor cells (Table 3). During fusion, 4.3% reconstructed oocyte showed zona dissolution and during culture, 26% showed one side notch, 21.7% showed both side notches, 4.3% reached to 2-cell stage and only 4.3% reached to morula stage (Fig. 2).

There is a progression in the stage of development, from embryos to adults, in terms of the level of differentiation in the various mammalian cell types that have been reprogrammed by NT procedures and yielded viable offsprings. In the majority of reports, donor cells of embryonic, foetal and adult origin, which were successfully used for NT were either quiescent (GO) *in situ* or were induced into GO by serum deprivation³.

Both AF and FF cells were cultured in monolayer after isolating the individual cells from the skin by enzymatic treatment. Isolation of FF cells took much lesser time (30 min) than AF cells (1½ hrs. FF cells got attached with the plastic surface of culture well

much earlier (3-6 hrs) than the AF cells (24-36 hrs). FF cells were also found in confluent state much earlier (2-3 days) than AF cells (7-8 days). Cytochalasin-B blocked cells were used for reconstruction of embryos. During primary culture 10% serum (FCS) was used and subsequently 2-3% serum was used during subculture. Initially fibroblast cells were much healthier and spindle shaped but subsequently during subculture (after 4-5 passage), they became tapered. FF cells survived longer time than that of AF cells, but due to aneuploidy increase, the cells were not able to maintain the surface attachment and became dead and got sloughed-off. The percentage of sloughing-off was more in AF cells as compared to the FF cells. Serum starvation, widely used to render cultured cells into quiescent GO cell cycle stage, can induce DNA degradation in a high proportion of cells¹¹. This, in turn, may cause impaired development of NT embryos. The variety of cultured FF/AF cells were used in NT, e.g. cumulus cells¹² and mural granulosa cells¹³ to skin fibroblasts¹⁴ and mammary gland epithelial cells^{15,16} though the overall efficiency of producing offspring is still very low (1%). Wells *et al*¹³ were able to increase the efficiency for the production of calves derived from adult mural granulosa cells. The transferred NT-blastocyst (10%) developed to term and the overall efficiency was 2.8%. Zakhartchenko *et al*¹⁷ cultured FF cells from bovine fetus, until sub-confluence (usually 2-3 days) and then frozen in 10% (v/v)

Table 2—Cytochalasin-B treated cell cycle arrest in FF & AF cells

Stage of cell cycle	Total no. of cells screened each time	Results						
		0 hr	30 min	1 hr	1 ½ hrs	2 hrs	4 hrs	8 hrs
FF cells arrested/Blocked in metaphase stage	200	19(9.5)	128(64)	170(85)	176(88)	180(90)	-	-
AF cells arrested/Blocked in metaphase stage	200	16(8)	-	-	-	19(9.5)	52(26)	133(66.5)

Figures under parenthesis show the percentage

Table 3—Development of early stage embryos by using Cytochalasin-B blocked donor cells

Type of donor cells	Total no. of oocytes matured	% of oocytes			Development, %				
		Enucleated/	Fused	Activated reconstructed	ZD	N1	N2	2 Cell	Morula
Cytochalasin-B blocked FF cells	76	69.7 (53)	43.4 (23)	43.4 (23)	4.3	26.0	21.7	4.3	4.3

N1-oneside notch, N2-bothside notch, ZD-Zona dissolved

Figures under parenthesis indicate no. of observations

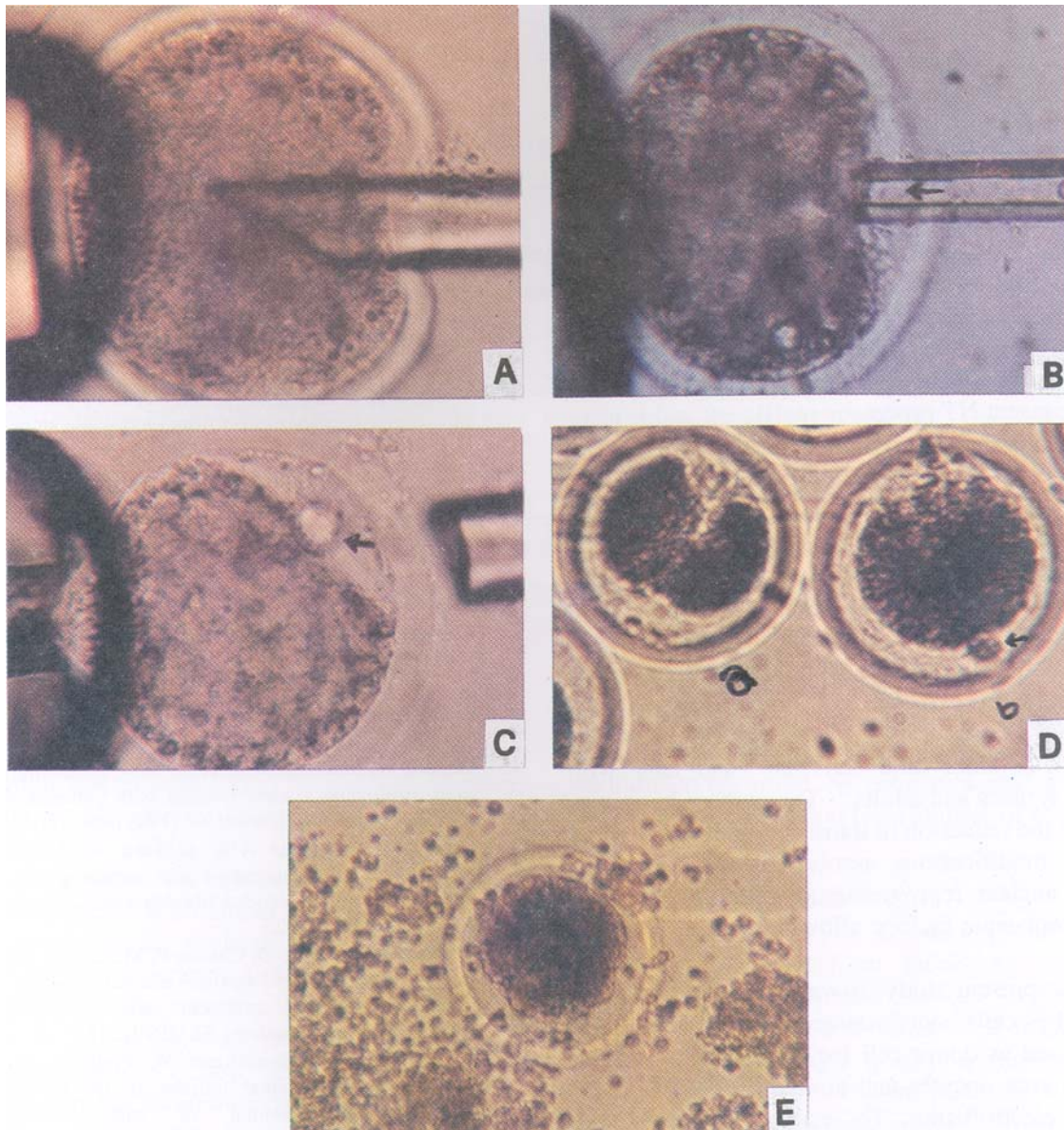


Fig. 2—A. Enucleation of matured oocyte by enucleation pipette piercing the zona of the oocyte held by holding pipette ($\times 400$); B. Transfer of donor cell ($\rightarrow$) by transfer pipette into the perivitelline space of enucleated oocyte ($\times 400$); C. An enucleated oocyte with donor cell ($\rightarrow$) at the perivitelline space ($\times 400$); D. Couplets constructed, electrofused and cultured for 48 hrs: a) developed to 2-cell embryo, b) donor cell did not fuse with cytoplasm ($\times 200$); & E. Couplets constructed, electrofused and cultured. Developed to compact morula with granulosa feeder cells support ($\times 200$).

DMSO (dimethyl sulfoxide) in culture medium and stored in liquid nitrogen. The frozen cells were thawed and cultured for up to four passages and used for nuclear transfer directly or after serum starvation. However, they have produced 20% and 39% blastocyst in non-starved and starved fibroblast cells respectively when used as donor cell.

The cell cycle stage of the donor cell affects the extent of embryo development after NT. The matured

oocytes arrested in the second metaphase in meiosis. Therefore, to keep the compatibility, the donor cells also arrested in the metaphase stage by using cytochalasin-B. When the donor cell is fused to the recipient oocyte, which is arrested in the second metaphase in meiosis, the nuclear envelope breaks down and the chromosomes condense until the oocyte is activated. This condensation phase has been shown to cause chromosomal defects in donor cells

undergoing DNA synthesis. Donor cells in the G1 phase of the cell cycle (before DNA synthesis), however, condense normally and support a high rate of early development¹. There was no significant difference among passages when the 1-6 passages of cumulus cells were used in nuclear transfer¹⁸. However, no blastocyst was produced in preactivated group and 29.9% blastocyst produced in delayed activated group. The production of live lambs occurred only from 1-3 passage cells without cell cycle synchronization, but no lambs were produced from 6-13 passages unless cell cycle was synchronized¹⁹.

Despite the use of quiescent cultured fibroblast cells, the present NT process is inefficient and is associated with continual early embryonic stage arrest. In goat, 43% cleavage achieved by culturing in mSoFaa media and the culture condition¹⁰ was 38-39°C, 5% CO₂, 7% O₂, and 88% N₂. In earlier studies, lambs were born following NT with unsynchronized cells from primary embryonic cell cultures with pre-activated cytoplasts²⁰. Viable offsprings were produced from cultured cells, previously starved of nutrients, forcing them to exit the cell cycle and enter a presumptive GO state, used for NT. Initially this was achieved with embryonic cell line¹⁹ and later with cells from fetuses and adults²¹. The authors have suggested that the reduction in transcriptional activity and chromatin modifications associated with GO may facilitate nuclear reprogramming upon exposure to oocyte cytoplasmic factors, allowing normal development.

From the present study, it was concluded that 3-5 passaged FF cells synchronized by cytochalasin-B could be used as donor cell for the reconstruction of enucleated goat oocytes and around 300 V would be better for electrofusion. To enable the commercial application of this technology, the efficiency of the current NT process using cultured cells needs to be increased.

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