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HETEROGENEOUS NUCLEAR TRANSFER EMBRYOS RECONSTRUCTED BY BOVINE OOCYTES AND CAMEL (*CAMELUS BACTRIANUS*) SKIN FIBROBLASTS AND THEIR SUBSEQUENT DEVELOPMENT

HUANMIN ZHOU[1] AND ZHENHUA GUO

College of Bioengineering, Inner Mongolia Agricultural University, Hohhot 010018 China

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SUMMARY

This study reconstructed heterogeneous embryos using camel skin fibroblast cells as donor karyoplasts and the bovine oocytes as recipient cytoplasts to investigate the reprogramming of camel somatic cell nuclei in bovine oocyte cytoplasm and the developmental potential of the reconstructed embryos. Serum-starved skin fibroblast cells, obtained from adult camel, were electrically fused into enucleated bovine metaphase II (MII) oocytes that were matured in vitro. The fused eggs were activated by Inomycin with 2 m*M*/ml 6-dimethylaminopurine. The activated reconstructed embryos were co-cultured with bovine cumulus cells in synthetic oviduct fluid supplemented with amino acid (SOFaa) and 10% fetal calf serum for 168 h. Results showed that 53% of the injected oocytes were successfully fused, 34% of the fused eggs underwent the first egg cleavage, and 100% of them developed to four- or 16-cell embryo stages. The first completed cleavage of xenonuclear transfer camel embryos occurred between 22 and 48 h following activation. This study demonstrated that the reconstructed embryos underwent the first embryonic division and that the reprogramming of camel fibroblast nuclei can be initiated in enucleated bovine MII oocytes.

Key words: camel skin fibroblast; bovine oocyte; reconstructed embryo; heterogeneous nuclear transfer.

INTRODUCTION

Production of cloned animals by somatic cell nuclear transfer has been successfully achieved in many mammalian species (Wilmut et al., 1997; Kato et al., 1998; Wakayama et al., 1998; Baguisi et al., 1999; Wells et al., 1999; Onishi et al., 2002; Shin et al., 2002; Galli et al., 2003; Woods et al., 2003; Zhou et al., 2003). Moreover, the delivery and pregnancy of embryos reconstructed by xenonuclear (cross-species) transfer using somatic cells have been reported in wild animals (White et al., 1999; Loi et al., 2001; Vogel, 2001). The generation of viable cloned offspring from different somatic cells demonstrated that terminally differentiated mammalian somatic cells could be dedifferentiated and recovered totipotency within enucleated oocytes. Therefore, somatic cell nuclear transfer technology has potential for practical application in the in vitro production of embryos, animal improvement, and the preservation of endangered species (Campbell, 1999; Graham and Bruce, 1999; White et al., 1999; Loi et al., 2001; Robl, 1999; Vogel, 2001).

So far, xenonuclear transfer in camels has not been reported. In addition, the camel is an important livestock species in China and employed both for transportation and for fieldwork as well as for milk and/or meat production. However, the camel population has been reduced rapidly because of the substitution of agricultural machinery in the past two decades. Since there is limited availability of camel oocytes for in vitro production but an abundant supply of bovine oocytes, the use of bovine oocytes as recipient cytoplasm would be an alternative approach for camel somatic cell nuclear transfer research. Consequently, this study attempted to reconstruct heterogeneous embryos using camel skin fibroblast cells as the donor karyoplasts and enucleated bovine oocytes as the recipient cytoplasts for examining the reprogramming of camel somatic cell nuclei in enucleated bovine oocyte cytoplasm and the developmental potential of the xenonuclear transfer embryos and determining the suitability of bovine oocytes as host cytoplasts for camel fibroblast cells.

MATERIALS AND METHODS

Preparation of donor cell line. Camel fibroblast cell line was derived from an adult camel. Skin tissues were collected from the edge of the ear. The skin sample was scraped off hair, sterilized with tincture of iodine, and stored in a Thermos filled with physiological saline at 37° C, then immediately transported to the laboratory. Keratoderma and connective tissues of the skin were removed with optical scissors. The remaining cutis tissues were washed three times in phosphate-buffered saline (PBS) with penicillin (100 IU/ml) and streptomycin (100 IU/ml) and placed in a Petri dish containing Dulbecco's modified Eagles's medium (DMEM)-F12 (Gibco BRL) supplemented with 10% fetal calf serum (FCS) (Gibco BRL), penicillin (100 IU/ml), and streptomycin (100 IU/ml). Then they were cut into small pieces (1 mm^3). The tissue pieces were transferred to a 25-ml culture flask containing 2 ml DMEM-F12 culture medium supplemented with 10% FCS, 100 IU/ml penicillin, and 100 IU/ml streptomycin and then incubated in humidified atmosphere of 5% CO_2 at 37° C for 3 d.

[1] To whom correspondence should be addressed at E-mail: huanminzhou@263.net

Individual cells were recovered when a fibroblast monolayer formed around the tissue explants attached on the culture flask bottom and cell proliferations reached 80% confluence. The culture medium and unattached epithelial cells were discarded. The attached fibroblast cells were washed with DMEM-F12 culture medium one time. Then they were digested by 0.25% trypsin in D-Hanks (Gibco BRL) at 37° C for 5 min and dispersed by pipetting. Digestion was terminated by adding 1 ml DMEM-F12 culture medium containing 10% FCS into the culture flask. The cell suspensions were transferred to a centrifuge tube and centrifuged at 100 × *g* for 10 min. Subsequently, the recovered cells were subcultured as described previously for four to six passages, then frozen in DMEM-F12 culture medium containing 10% dimethyl sulfoxide and 20% fetal calf serum (FCS) (Gibco BRL) in liquid Nitrogen (LN_2).

The fibroblast cells were thawed at 37° C, cultured for four to six passages, and used for nuclear transfer. The donor cells were serum-starved in DMEM-F12 supplemented with 0.5% FCS for 3–5 d before being used for nuclear transfer. Then the cells were detached from culture dishes by brief exposure to 0.25% trypsin-0.02% ethylenediamine-tetraacetic acid and subsequently washed five to seven times in H-M199 culture medium supplemented with 10% FCS and then transferred into a micromanipulation drop for use.

Oocyte maturation. Bovine ovaries were collected at a local slaughterhouse and transported to the laboratory within 2 h in a Thermos filled with physiological saline containing 1% gentamycin at 25–30° C. Cumulus oocyte complexes (COCs) were extracted from antral follicles of 2–5 mm in diameter with a 12-gauge needle equipped to a 10-ml disposable syringe contained 1 ml TCM 199 supplemented with 50 mg/ml heparin and 0.5% FCS. Only the oocytes with homogeneous cytoplasm and compassed by more than three layers of compact cumulus cells were chosen for in vitro maturation. They were washed three to five times in maturation culture medium composed of TCM 199 (Gibco BRL) supplemented with 20% FCS, 10 μg/ml follicle-stimulating hormone (Ning Bo Hormone Factory, Ning Bo, China), 20 μg/ml luteinizing hormone (Ning Bo Hormone Factory), and 1.5 μg/ml 17-β estradiol (Ning Bo Hormone Factory). A group of 20–30 selected COCs were matured in a 50-μl microdrop of maturation culture medium under mineral oil (Sigma) in a 35-mm Petri dish in a humidified atmosphere of 5% CO_2 at 38.5° C for 22 h. Then the expanded cumulus cells of COCs were removed by digestive treatment with 0.2% hyaluronidase and pipetting. The oocytes with extruded first polar body (metaphase II [MII]) were selected for use as recipient cytoplasm in the nuclear transfer procedures.

Additional COCs aspirated from the follicles were used for the preparation of cumulus cells. Cumulus cells were released from COCs through repeated pipetting, then rinsed in TCM 199 culture medium and recovered for use in the culture.

Oocyte enucleation. Bovine MII oocytes were denuded free cumulus cells and incubated in 5 μg/ml Hoechst 33342 (Sigma) in H-M199 for 15 min for staining oocyte nuclei, then placed in a microdrop of H-M199 supplemented with 10% FCS and 5 μg/ml cytochalasin B (Sigma). They were enucleated by aspirating the first polar body and its surrounding cytoplasm. Successful enucleation was confirmed by fluorescent stain under an inverted contrast microscope equipped with a fluorescent optics system (Zeiss, Jena, Germany) at 200× magnification. These enucleated oocytes were used as recipient cytoplasts.

Nuclear transfer, fusion, activation, and subsequent culture. A donor cell with approximately 25 μm in diameter was selected and injected into the perivitelline space of an enucleated oocyte using a micromanipulator (Eppendorf, Hamburg, Germany). The fibroblast–cytoplasm couplets were washed and incubated in fusion medium, which consisted of 0.3 *M*/ml mannitol, 0.5 m*M*/ml Hepes, 0.05 m*M*/ml $CaCl_2$, 0.1 m*M*/ml $MgCl_2$, and 0.05% bovine serum albumin (Sigma) for 2 min. Then they were deposited in a fusion chamber between two platinum electrodes (0.1 mm apart) and filled with fusion medium. The cell fusion was induced by 2 d.c. electrical pulses of 1.6 KV/cm for 20 μs with an interval of 0.5 s. The fusion was ascertained by microscopic examination. The fused oocytes were activated immediately by exposure to 5 μ*M*/ml Inomycin in H-M199 for 5 min. After washing three times in SOF, they were incubated in synthetic oviduct fluid supplemented with amino acid (SOFaa) and 2 m*M*/ml 6-DMAP in a humidified atmosphere of 5% CO_2 at 38.5° C for 4 h. Subsequently, a cohort of 6–10 activated nuclear transfer embryos were seeded in a 20-μl droplet of SOFaa containing 10% FCS covered with mineral oil in a 35-mm Petri dish and cocultured with ovine cumulus cells in a humidified atmosphere of 5% CO_2 at 38.5° C for seven consecutive d. Half volume of the medium was replaced by an equal volume of fresh medium every 3 d. The cultured reconstructed embryos were visually assessed for the first completion cleavage at 48 h of culture and for subsequent developmental stages at 168 h of the culture using an inverted phase-contrast microscope.

Statistical analysis. The fusion rate was expressed by the number of fused oocytes as a percentage of the total of injected oocytes. The cleavage rate was expressed by the number of cleaved eggs at 48 h of the culture as a percentage of the total activated eggs at the beginning of the culture. The blastocyst rate was expressed by the number of blastocysts at 168 h of the culture as a percentage of the total of cleaved eggs at 48 h of culture.

Results

Camel skin fibroblast cells grew rapidly and proliferated consistently in culture. They were capable of growing and exhibited normal morphology in serum starvation culture (Fig. 1). Their morphological characteristics and normal number of chromosomes (2n = 72) were normally maintained (Fig. 2) after they underwent over nine passages of culture.

The results of cell fusion, cleavage, and subsequent development of embryos reconstructed using camel skin fibroblasts as donor cells and enucleated bovine oocyte as host cytoplasm are summarized in

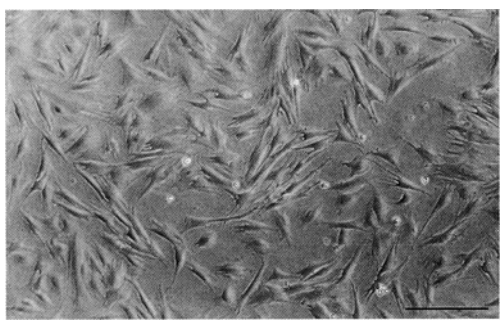

FIG. 1. Camel fibroblast cells during serum starvation culture for 3 d. Bar represents 100 μm.

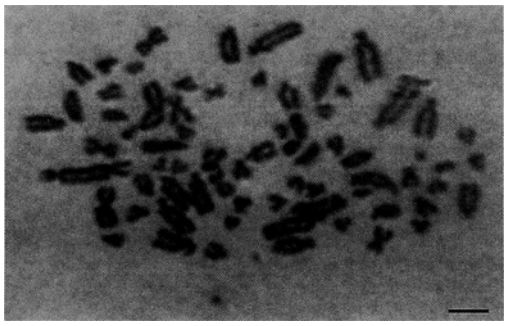

FIG. 2. Chromosomes (2n = 72) of camel skin fibroblast cultured for nine passages. Bar represents 100 μm.

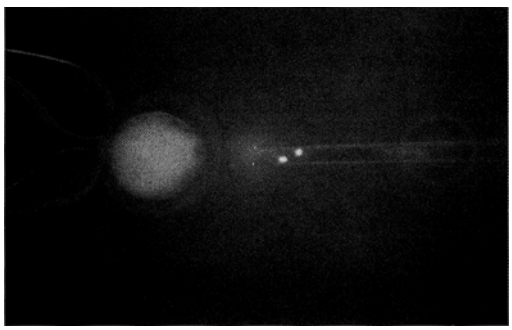

FIG. 3. Enucleated oocytes stained with fluorescent dye. Both first polar body and nuclear were aspirated out of oocyte. Bar represents 100 μm.

Table 1. The fusion rate of fibroblast cells with enucleated bovine cytoplasm was 53%. The first completed cleavage of xenonuclear transplantation camel embryos occurred between 22 and 48 h following activation. The results indicated that 34% of the fused oocytes underwent the first embryonic division, 71% of them developed to the two- or four-cell embryo stage, and 29% of them developed to the 8- or 16-cell embryo stage (Fig. 4). However, no reconstructed embryo developing to the blastocyst stage was observed (Table 1).

DISCUSSION

The successful fusion of donor cells into recipient oocytes is a prerequisite for the reconstruction and development of nuclear transfer embryos. This study demonstrated that an induction by electrofusion yielded a 53% rate of fusion of fibroblast cells into enucleated bovine oocytes. However, this result is lower than that obtained in other mammalian species where homogeneous or heterogeneous nuclear transfer embryos were constructed by different types of somatic cells with enucleated bovine oocytes (Cho et al., 2002; Saikhum et al., 2002). These differences may be attributed to the different resources of somatic cells. It has been reported that there were significant differences in fusion rate among somatic cells derived from various tissue types in mammalian species (Cho et al., 2002; Saikhum et al., 2002; Lee et al., 2003). The fusion between donor cells and host oocytes is affected mainly by the contact of the donor cells with the recipient oocytes, the difference of cell membranes between the donor cells and the recipient oocytes (Dominko et al., 1999), the integrity of the cell membrane (Cho et al., 2002), and the size of the donor cells (Dominko et al., 1999). Overall comparisons suggested that the difference in fusion rate might be species-related between the donor cells and the recipient cytoplasts.

TABLE 1

FUSION AND EMBRYONIC DIVISION OF ENUCLEATED BOVINE OOCYTES RECONSTRUCTED WITH CAMEL SKIN FIBROBLAST CELLS

Oocytes injected	Oocytes fused (%)	Embryos cleaved (%)	2–4-cell embryos (%)	8- or 16-cell embryos (%)	Blastocyst (%)
132	70 (53)	24 (34)	17 (24)	7 (24)	0 (0)

This study indicated that 34% of camel reconstructed embryos completed their first embryonic division and that 29% of them developed to the 8- or 16-cell embryo stage, demonstrating that bovine MII oocyte cytoplasm was capable of activating the genome after transplantation of a donor karyoplast. Since chromosomes of bovine oocytes were removed and confirmed by exposure to ultraviolet light (Fig. 3), it is reasonable to assume that the early development of reconstructed embryos resulted from the reprogramming of camel donor nuclei. This suggested that the nucleo-cytoplasmic interaction between camel fibroblast cells and enucleated bovine MII oocytes benefited the first mitotic cycles of the reconstructed embryos. It has been believed that the bovine oocyte cytoplasm could be a universal recipient for the development of the introduced fibroblast cells from various mammalian species (Dominko et al., 1999). However, although 34% of the reconstructed embryos underwent the first cleavage and some differentiated to the 16-cell embryo stage, none of them developed to the blastocyst stage in the present study. Their competence of development was similar to that achieved in horse, where there was a very limited potential for further development in embryos reconstructed by fibroblast cells and enucleated bovine oocyts after the first mitotic cycle (Sansinena et al., 2002). Also, their developmental ability was poorer than that of other animal species where the reconstructed embryos had developed to the blastocyst stage (Cho et al., 2002; Saikhum et al., 2002; Lee et al., 2003). In the present study, the poor potential of development of reconstructed embryos to the blastocyst stage during culture period may be related to several factors: some essential agents might be insufficient in the recipient oocytes matured in vitro for cell mitotic progression of the cloned embryos after the early embryonic division, critical errors may occur during the reprogramming of the cloned embryo that could negatively affect the cell division, the

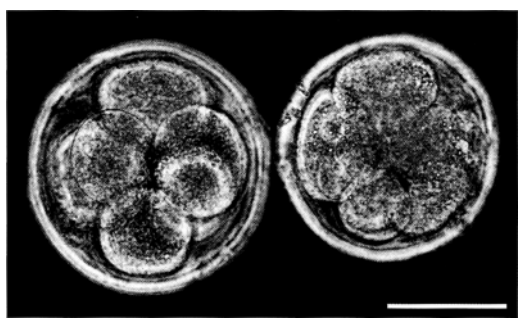

FIG. 4. Embryos reconstructed from camel skin fibroblast cell and enucleated bovine oocyte developed to the 8- or 16-cell stage. Bar represents 100 μm.

integrity of donor cell membrane might be damaged because of serum starvation or frozen-thaw treatments that could cause the donor cells to degenerate and result in damaged chromosomes that could not be remodeled by the recipient cytoplasm, or morphological or genetic elements of donor somatic cells during cell preparation might be changed. It has been demonstrated that morphology of donor cells apparently affected the condensation of chromosome and the formation of nuclei in the reconstructed embryos (Tao et al., 1999), and chromatin modifications in some donor cells may interfere with their reprogramming following fusion into the host cytoplasm (Campbell et al., 1996).

Skin fibroblast cells were recommended to be used as somatic donor cells, and long-term culture did not seem to compromise their cloning competence (Dominko et al., 1999). In the present study, camel skin fibroblast cells could maintain normally morphological characteristics and chromosome numbers after prolonged culture. Their nuclei could be reprogrammed in enucleated bovine oocytes matured in vitro. Nevertheless, the cell cycle of donor somatic cells was not screened in serum starvation culture. It remains unknown whether the pattern of donor cells progressed to the G_0/G_1 phase of the cell cycle in passage culture. In addition, the effect of in vitro and in vivo maturation of bovine oocytes on the reprogramming of somatic donor cells and the development of reconstructed embryos is still unclear. More experiments are needed to investigate the critical factors affecting the developmental competence of the cloned embryos.

In conclusion, the reprogramming of camel dermal fibroblast cell nuclei can be initiated in bovine enucleated oocyte cytoplasm. The reconstructed embryos underwent the first embryonic division, but their further developmental competence was limited. Further research will focus on establishing a full-term development of the xenonulear transfer embryos.

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