Vitrification of water buffalo (Bubalus bubalis) fetal skin fibroblast cells

C R Meena and S K Das*
Embryo Biotechnology Centre, Division of Animal Biotechnology, National Dairy Research Institute
Karnal 132 001, India
1National Dairy Research Institute, Eastern Regional Station, Kalyani, Nadia 741 235, India

Received 24 September 2004; revised 24 May 2005; accepted 16 July 2005

A method was developed for the cryopreservation of buffalo fetal skin fibroblast cells by vitrification. Skin cells were isolated from 1–2-month-old fetuses obtained from an abattoir, by enzymatic digestion (0.5% w/v trypsin + 0.05% w/v collagenase in DPBS) for 15–20 min. They were washed 5–6 times with DPBS and then once with RPMI-1640 + 10% FBS by centrifugation at 600 rpm. The cells were then cultured in the same medium in a CO₂ incubator (5% CO₂ in air) at 38.5°C for 3 d. After 3 days, the fibroblast cells from the monolayer were harvested by treating with trypsin-versene solution. They were equilibrated and loaded into 0.25 mL French straw, containing 0.5 M sucrose solution in DPBS. The straws were sealed and precooled by keeping them in liquid nitrogen (LN₂) vapour for 1–2 min, following which these were plunged into LN₂. After 5 days of storage the straws were warmed quickly by transferring them to a water bath at about 20°C for 15–20 sec. The cells were collected by centrifugation and plated in 25 cm² culture flasks with 5–6 mL of culture media and incubated at 38.5 ± 1°C with 5% CO₂ in humidified (95%) incubator. The fibroblast cells growth started within 3 h of culture set-up and on day 3 the monolayer formation occurred.

Keywords: buffalo, cryopreservation, fetal skin fibroblasts, vitrification

IPC Code: Int. Cl. A23B4/09

The application of somatic cells in the nuclear transfer programme for the production of cloned embryos in buffalo is increasing day-by-day. The success rate of nuclear transfer using somatic cells is inefficient, and only 1–2% of reconstituted sheep, cow, mouse, and goat embryos develop to term. 1-5 Fetal fibroblasts have been frequently used as donor cells and the rate of development of reconstituted embryos is thought to be enhanced when donor nuclei are at the G0 phase of cell cycle. The availability of the specific cells of a particular stage has great importance during the time of reconstruction of oocytes. The in vitro maturation procedure has been successfully used for routine production of matured oocytes from slaughter house ovaries in buffalo and the culture of skin fibroblast cells also been successfully developed. The practical application of these techniques is, however, severely hampered when the specific cell type of a particular stage and passage required for the reconstruction of in vitro matured enucleated oocytes is not available. The cryopreserved cells could increase their instant availability for a wide application of reproductive technologies in the buffalo including somatic cell nuclear transfer.

In the recent past cryopreservation techniques have been substantially improved and applied in different fields like oocyte preservation, embryo preservation. However, many cell lines/cells/oocytes/embryos are still being frozen by conventional equilibrium methods involving controlled cooling rates, which require the use of expensive machineries, which also form ice crystals.

Vitrification has been used successfully for the cryopreservation of oocytes and embryos. There is, however, no report available on the cryopreservation of water buffalo fetal skin fibroblast cells by vitrification. The present study, therefore, was undertaken to develop a method for cryopreserving buffalo fetal skin fibroblast cells by vitrification, and to examine the growing capability of vitrified cells after thawing.

The buffalo gravid uterus were collected from slaughterhouse and transported to the laboratory within 3 to 4 h. The uterus was washed several times with lukewarm water. Then the fetus was taken out from the uterus aseptically and washed with lukewarm normal saline supplemented with gentamycin. The skin sample was collected from fetus and washed with calcium and magnesium free Dulbecco’s phosphate buffered saline (DPBS). Skin samples were chopped in small pieces and washed 5–6 times with DPBS; finally the cells were isolated by enzymatic digestion in DPBS supplemented with 0.5% (w/v) trypsin and 0.05% (w/v) collagenase. After 15 min of digestion, cells were separated individually and released into the media (Fig. 1A). The released cells were washed 5-6
times (600 rpm/10 min) with DPBS and finally with the culture media (RPMI-1640+10% FBS) was supplemented with gentamycin and amphotericin-B. The cells were then plated in 25 cm² culture flask with 5-6 mL of culture media and incubated at 38.5±1°C with 5% CO₂ in humidified (95%) incubator for monolayer formation.

After 3 days of primary culture set-up the confluence formation occurred. For harvesting the media was replaced with 3 mL of trypsin-versene solution and incubated for 3-4 min. The attached growing cells became loose and got detached from the plastic surface of the flask. The solution along with cells collected in 15 mL centrifuge tube and washed with culture media 2-3 times (600 rpm/5 min) and the cell pellet was dissolved in 1 mL of culture media. The harvested fibroblast cells were equilibrated in the equilibration solution for 1-2 min and then transferred into the vitrification solution (VS) at room temperature (22-25°C). The VS (25% v/v ethylene glycol (EG) + 25% v/v dimethyl sulphoxide (DMSO) + 50% DPBS + 0.33 mM sodium pyruvate + 5.56 mM glucose + 4 mg/mL BSA) and equilibration solution (50% vitrification solution + 50% DPBS) were prepared as described by Das et al. The cells were then immediately loaded into 0.25 mL French straw, containing 0.5 M sucrose solution in DPBS in both the side of the VS. The straws were sealed with hot forceps, and were precooled by keeping them in liquid nitrogen (LN₂) vapour at a height of about 3-5 cm from the level of LN₂ surface for 1-2 min, following which these were plunged into LN₂. After 5 days of storage the straws were warmed quickly by transferring them into a water bath at about 20°C for 15-20 sec. The contents were expelled into an empty plastic dish, and then mixed with 0.5 M sucrose solution in DPBS for equilibration. The cells were collected by centrifugation and washed with culture media and plated in 25 cm² culture flask with 5-6 mL of culture media and incubated at 38.5±1°C with 5% CO₂ in humidified (95%) incubator.

Within 2-3 h of primary culture set-up, the fibroblast cells started growing (Fig. 1B) and on day-3 the confluence formation occurred (Fig. 1C). Following vitrification-warming, the recovered cells were morphologically normal (Fig. 1D) and after incubating, the fibroblast cells growth started within 3 h when cells were equilibrated for 2 min and 4.5 h when cells were equilibrated for 1 min after the culture set-up. The monolayer formation occurred on day 3 (Fig. 1E) in case of 2 min equilibration time and on day 4 in case of 1 min equilibration time. However, there was no difference in time required for monolayer formation in cells vitrified after 2 min of equilibration or freshly isolated cells (Table 1).

The results show that buffalo fetal skin fibroblast cells can be vitrified by a simple and rapid technique in a solution of EG and DMSO. Several of cryoprotectants [glycerol, propylene glycol (PG), DMSO, polyethylene glycol (PEG), 1-2 propanediol] have been used in different combinations for vitrification of mammalian oocytes and embryos. Among these, EG is an effective cryoprotectant because of its higher permeation into the cells for vitrification and faster removal during dilution of post-thawing, as its molecular weight is lower than that of glycerol and PG. EG has been found to be less toxic than glycerol and PG to mouse embryos and the post-vitrification survival of basic embryos has been found to be much higher in EG than in either a combination of DMSO, PG and PEG or a combination of glycerol and PG.
The period of exposure of cells to cryoprotectants for vitrification is of critical importance. Normally, cells are equilibrated in a solution containing low concentration of cryoprotectants before very short exposure to vitrification solution. In this study, exposure of cells to the equilibration solution for 1 min resulted little late initiation of growth as compared to 2 min equilibration time.

In conclusion, the results of the present study suggest that it is possible to cryopreserve water buffalo fetal skin fibroblast cells by vitrification using a combination of EG and DMSO with an exposure time of 2 min, which could be used as donor nuclei in the nuclear transfer programme. Morphologically, the cells are equally normal and culturable. However, additional studies are needed to evaluate the developmental competence and normality of reconstructed embryos produced from nuclear transfer of vitrified fibroblast cells to enucleated oocytes.

Acknowledgement

The authors are thankful to the Director, National Dairy Research Institute, Karnal for providing necessary facilities to carry out this work.

References