Vitrification of camel skin tissue for use as a resource for somatic cell nuclear transfer in *Camelus dromedarius*

Young-Bum Son¹ · Yeon Ik Jeong¹ · Yeon Woo Jeong¹ · Xianfeng Yu² · Lian Cai¹ · Eun Ji Choi¹ · Mohammad Shamim Hossein¹ · Alex Tinson³ · Kuhad Kuldip Singh³ · Singh Rajesh³ · Al Shamsi Noura³ · Woo Suk Hwang¹*

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Animals with superior genetics could be reproduced by somatic cell nuclear transfer (SCNT). Generally, for SCNT viable cell lines are established from living animals (Jeong *et al.* 2020). However, complication arises when animals die suddenly due to a disease or accident (Jeong *et al.* 2013). A suitable tissue cryopreservation method that can preserve tissue integrity is important. The cryopreservation is a prominent method for preserving animal genetic resources, and autologous cells could be obtained from cryopreserved tissue which has numerous clinical applications (Buck *et al.* 1981; Taylor *et al.* 2019). Therefore, studies on tissue cryopreservation in several species, such as humans, rats, sheep, and dog, have been reported (Ishijima *et al.* 2006; Wang *et al.* 2008; Courbiere *et al.* 2009; Deng *et al.* 2009). However, studies have not been reported on the establishment of cells from cryopreserved tissues of *Camelus dromedarius* (camel). In addition, limited studies have been reported on cloning through the SCNT technique using cells established from cryopreserved tissues without cryoprotectants (Hoshino *et al.* 2009; Zhang *et al.* 2012; Jeong *et al.* 2020).

Tissue cryopreservation is largely a slow-freezing and vitrification method. In general, programmed slow freezing is used for tissue freezing (Santos *et al.* 2007; Dalman *et al.* 2017; Lee *et al.* 2019). Vitrification is a simple, inexpensive and rapid method for cryopreservation (Dalman *et al.* 2017; Lee *et al.* 2019; Santos *et al.* 2007; Wang *et al.* 2008). It could replace the slow freezing techniques as it prevents the formation of ice crystals due to an ultrarapid cooling procedure (Amorim *et al.* 2011). For vitrification of tissues, intracellular cryoprotectants such as dimethyl-sulfoxide (Me2SO), ethylene glycol (EG), and glycerol are mainly used (Santos *et al.* 2007; Amorim *et al.* 2011). Among these, EG has the lowest cytotoxicity and a rapid permeability across the cell membrane (Bautista and Kanagawa 1998; Eto *et al.* 2014; Kuleshova *et al.* 1999). Furthermore, EG leads to thinning of the phospholipid biolayers and diffuses through them (Hughes and Mancera 2014). Therefore, tissue vitrification using EG has been reported in various mammals (Isachenko *et al.* 2003; Yeoman *et al.* 2005; Gandolfi *et al.* 2006). However, studies on cryopreservation of tissue, except for ovarian tissue, are very limited, and no studies on tissue vitrification using EG in camels have been reported. Therefore, the present study established cell lines from vitrified camel skin tissues, their characteristics were evaluated accordingly, and the developmental efficiency of the embryo was analyzed after performing SCNT using these cells as donor cells.

All animal work was performed according to the animal study guidelines which were approved by the ethics committee at the Management of Scientific Centers and Presidential Camels (Accession No: PC4.1.5). To preserve the genetic resources of a camel (male) that died suddenly, vitrification was performed after biopsy of the ear skin tissue. As a control for evaluating the effectiveness of the tissue vitrification method, ear skin tissues were collected from 6 different camels (three males and three females). The chemicals used in this study were purchased from Sigma (St. Louis, MO) unless otherwise noted. We adjusted the pH to 7.4 and the osmolality to 280 mOsm/kg for all media.
We performed tissue vitrification as previously described with minor modifications (Santos et al. 2007). Briefly, tissues were washed with DPBS (Life Technologies, Carlsbad, CA) containing 10 μg/mL penicillin/streptomycin solution (Invitrogen). After that, tissues were cut into small pieces (1 cm²) at 25°C. The fragments were exposed to Dulbecco’s modified Eagle’s medium (DMEM; Thermo Fisher scientific, Waltham, MA) supplemented with 20% EG and 10% FBS, 1% antibiotic-antimycotic, and 0.1% β-mercaptoethanol (Thermo Fisher Scientific) at 38°C in a humidified atmosphere of 5% CO₂. The number of viable cells and the cell proliferation capacity from vitrified and fresh tissues were analyzed as previously described with minor modifications (Legzdina et al. 2016; Shivakumar et al. 2016). Fibroblasts derived from both tissues were stained with 0.4% Trypan blue and the number of cells was counted every 2 d. Fibroblasts (2 × 10³) from both tissues were seeded into 6-well plates, and after every 72 h, the cells were counted using a hemocytometer. The population of doubling time (PDT) of the cells was calculated in accordance with the formula, PDT = log₂ × T/(logNH – log NI), where T is the culture time, logNH is the harvest cell number, and log NI is the initial cell number. Camel fibroblasts from fresh and vitrified skin tissues showed homogenous plate-adherent and spindle-like cell morphology (Fig. 1b, c). In the initial culture process, fibroblasts derived from fresh tissues showed enhanced growth patterns compared with those derived from vitrified tissues (Fig. 1d). However, cell proliferation assessed by PDT showed similar values of proliferation after passage 1 (Fig. 1e). Therefore, we assumed that fibroblasts isolated from vitrified tissues could be used as donor cells in the SCNT process, and analyzed the characteristics of the cells.

Cells lose their viability due to apoptosis induced during the tissue cryopreservation and thawing process (Amorim et al. 2011). This was one of the limitations of using tissue cryopreservation for securing adequate viable cells. Therefore, we cultured fibroblasts up to passage 3 for homogenization to use as donor cells for SCNT and analyzed cellular apoptosis that could have been induced by cryopreservation and the thawing of tissues. When cells from both fresh and vitrified tissues reached 80% confluence, senescence β-galactosidase (SA-β-gal) was analyzed using the senescence β-galactosidase

Figure 1. (a) H&E staining was conducted. Both tissues showed normal H&E staining. Scale bar = 800 μm. Morphology of camel fibroblasts from (b) fresh and (c) vitrified ear skin tissues (cryo), (d) cell proliferation analysis, and (e) population of doubling time assay (PDT). The fibroblasts from both groups showed a similar spindle-like morphology. The proliferative ability was decreased in the cryo group compared to the fresh group at the initiation of culture and at passage 1. However, there was no significant difference in the proliferative capacity between the two groups after passage 1. Scale bar = 200 μm. Data are represented by the mean ± SD of four independent experiments. Asterisks (*) indicate significant differences between groups (P < 0.05). (f) Cellular senescence was analyzed by SA-β-gal staining. The number of SA-β-gal positive cells was not different between the fresh- and the cryo groups. Scale bar = 200 μm.
stained. Finally, we stained detached cells (1 × 10^6) with 100 μg/ml oligomycin was loaded into the accompanying cartridge. Treatment with the drugs in the medium occurred at the time points specified (Fig. 2f). The OCR was monitored using a Seahorse Bioscience XF24 Extracellular Flux Analyzer. Each cycle was performed as follows: mix for 3 min, delay for 2 min, and then measure for 3 min. Basal respiration was evaluated before oligomycin injection, and proton leakage was calculated after oligomycin injection. ATP production was measured through the difference between basal respiration and proton leakage, and after FCCP injection, spare respiratory capacity was calculated through the difference between maximal respiration and ATP production. The OCR value was dramatically decreased after inhibition of the F0 ATP complex by treatment with oligomycin. After that, the OCR value was increased after the addition of FCCP, indicating that mitochondrial respiration was uncoupled in both groups. Our results revealed that the basal OCR indicating oxidative phosphorylation (OXPHOS) was not different in these groups (Fig. 2f). Furthermore, the values of basal respiration, spare respiratory capacity, proton leakage, and ATP production were also not significantly different between the groups (Fig. 2g). Overall, our data demonstrated that cells from vitrified tissues showed normal mitochondrial function compared to cells from fresh tissues.

We used fibroblasts derived from fresh and vitrified tissues as donor cells for SCNT. A total of 580 in vitro matured oocytes (96 ovaries) and 978 in vivo matured oocytes (78 oocyte donors) aged between 4 and 7 yr weighing 400 to 450 kg were used to evaluate the effect of donor cells on embryonic development after SCNT. The donors were injected with 5000 IU PMSG (Ceva, Libourne, France) and 500 μg of cloprostenol (Jurox, Rutherford, Australia) to stimulate the ovary as previously reported (Tinson et al. 2000). After that, the donors were injected with 100 μg gonadorelin...
acetate (Vetoquinol, Paris, France) 25 to 28 h before OPU was conducted. The oocytes were obtained by an Aloka Ultrasound Unit (Aloka) with a needle guide (Aloka, Tokyo, Japan) using 60-cm, 18-gauge lumen needle in the follicles. Ovaries were washed with 0.9% saline solution, and follicles were aspirated using an 18-gauge needle with a 10-ml disposable syringe. The homogenous cytoplasm enclosed by at least three layers of compact cumulus cells was collected and washed with DPBS containing 5 mg/ml bovine serum albumin (BSA; Thermo Fisher Scientific) and 1% antibiotic-antimycotic. For in vitro maturation (IVM), COCs were cultured at 38°C in 5% CO₂ in a humidified incubator for 42 h with IVM medium (IVF Bioscience, Falmouth, UK). After that, we conducted SCNT by the techniques as previously reported with minor modifications using in vitro and in vivo matured oocytes (Kim et al. 2012). The cumulus cells were denuded from oocytes by gentle pipetting with 0.1% hyaluronidase. The denuded oocytes were stained with 5 μg/ml bisbenzimide for 3 min and enucleated by aspirating the first polar body. After enucleation, a single donor cell was microinjected into the perivitelline space of the oocytes. These oocyte couplets were fused in fusion media containing 0.26 M mannitol, 0.1 mM MgSO₄, 0.5 mM HEPES, and 0.05% (w/v) BSA with two DC pulses of 1.8 kV/cm for 15 μsec using a BTX Electro Cell Manipulator (BTX Inc., San Diego, CA). The reconstructed embryos were treated with 5 μM ionomycin for 3 min and with 2.0 mM 6-dimethylaminopurine (6-DMAP) in BO-IVC (IVF Bioscience, Falmouth, UK) in a humidified incubator with 5% CO₂ at 39°C for 4 h. After activation, we cultured the embryos in 6 to 8 oil-covered droplets at 38°C in a humidified incubator with 5% CO₂ and 5% O₂ for 7 d. Our results showed that the rates of fused oocytes and cleaved embryos were similar in the fresh and cryopreserved groups (Table 2). The efficiency of blastocyst formation was slightly increased in the fresh group compared to the cryopreserved groups, but there

**Table 1.** List of primers used in the RT-qPCR analysis

| Gene name (symbol) | Primers sequence | Product size (bp) | Anneal. Temp (°C) |
|--------------------|------------------|------------------|-------------------|
| BCL2-associated X protein (BAX) | F: CTGAGCAGATCATGGAAGAC  
R: TACTGTCAGGTTCTATCC | 171 | 60 |
| BCL-2 antagonist/killer 1 (BAK) | F: TACGACTCAGAGTCCAG  
R: GCTGGTAGACATGTAGGG | 169 | 60 |
| Tumor protein 53 (p53) | F: CCACTACAAGAGGTCAGAG  
R: AGTGGATAGTGGTACAGTCA | 222 | 60 |
| Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) | F: TCACGCCCATCACAACAGT  
R: TCAGGCCCATCACAACAGT | 133 | 60 |

**Figure 2.** (a), (b) Analysis of the cell cycle and apoptosis in fibroblasts from fresh and vitrified ear skin tissues (cryo) at passage 3. No differences were observed between the fresh and cryo groups with respect to the portion of the cell cycle (a) and cellular apoptosis (b). (c) The mRNA expression levels of apoptosis-specific genes (Bax, Bak, and p53) were similar in both the fresh and cryo groups. Data are presented as the mean ± SD of four independent experiments. (d), (e) Analysis of mitochondrial membrane potential (ΔΨₘ) in fibroblasts from fresh and vitrified ear skin tissues (cryo). (d) Both types of fibroblasts were stained with JC-1 fluorescent dyes and DAPI. Scale bar = 100 μm. (e) The portion of red/green optical density was not significantly different between the fresh and cryo groups. Data are presented as the mean ± SD of four independent experiments. (f), (g) Oxygen consumption rate (OCR) assay of fibroblasts from fresh and vitrified ear skin tissues (cryo). (f) The cryo group showed similar mitochondrial respiration compared to the fresh group. (g) The values for basal respiration, proton leakage, spare respiratory capacity, and ATP production were similar in both groups. The data point in OCR represents the mean ± SD; n = 10 wells in independent experiments.
was no significant difference (Table 2). The efficiency of fusion, cleavage, and blastocyst formation showed similar patterns in both the in vitro and in vivo matured oocytes (Table 2). Furthermore, similar to previous studies, the in vivo matured oocyte group showed higher cleavage and blastocyst formation rates than the in vitro matured oocyte group (Wani et al. 2018). Therefore, we confirmed that the source of oocytes was not related to the effects of the donor cells.

In conclusion, fibroblasts from vitrified tissues showed decreased proliferation compared with cells from fresh tissues in the initial culture process but showed similar patterns after passaging. Furthermore, our data showed similar values of mitochondrial metabolism, and the expression patterns of the cell cycle and apoptosis were also similarly observed after homogenization. Therefore, we determined that cells from vitrified tissues were suitable donor cells for SCNT, and confirmed that the efficiency of embryo development was similar to that of the fresh group. Taken together, camel SCNT embryos using cells from vitrified tissues were successfully developed to the blastocyst stage. These results suggested new approaches to tissue vitrification in preserving genetic resources.

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Table 2. Effect of donor cells on the fusion rate and efficiency of embryo development of somatic cell nuclear transfer using in vitro and in vivo matured oocytes

| Donor cells | Source of oocytes   | No. of oocyte Reconstructed oocytes | Fused (%) | Cleaved (%) | Blastocyst (%) |
|-------------|--------------------|------------------------------------|-----------|-------------|---------------|
| Fresh       | In vitro matured oocytes | 301                                | 213 (72.13 ± 3.86) | 136 (65.83 ± 5.60)* | 43 (22.55 ± 2.83)* |
| Cryo        | In vitro matured oocytes | 279                                | 196 (71.38 ± 1.86) | 122 (62.47 ± 2.89)* | 41 (20.69 ± 1.16)* |
| Fresh       | In vivo matured oocytes  | 507                                | 385 (75.85 ± 1.32) | 285 (76.93 ± 1.98)* | 191 (45.89 ± 1.85)* |
| Cryo        | In vivo matured oocytes  | 471                                | 348 (75.79 ± 2.26) | 292 (74.91 ± 3.13)* | 183 (44.97 ± 3.05)* |

Data are represented by the mean ± SE of four independent experiments. Lettered subscripts indicate statistical differences between groups (P < 0.05)
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