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Reproductive stage-dependent effects of additional cryoprotectant agents for the cryopreservation of stallion germ cells

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ABSTRACT

The main objective of this study was to evaluate the efficacy of an additional cryoprotectant in 10% dimethyl sulfoxide (DMSO) on cryopreserving germ cells from stallions at different reproductive stages. Testicular samples were obtained from pre-pubertal (1–1.5 yr, n = 6) and post-pubertal (3–7 yr, n = 5) stallions. Germ cells were isolated using a two-enzyme digestion procedure and cryopreserved in minimal essential medium alpha containing 10% fetal bovine serum and 10% DMSO with or without addition of trehalose (50, 100, or 200 mM) or polyethylene glycol (PEG, 2.5, 5, or 10%). Viability, cell population, and viable population were assessed after 1 and 3 months of cryopreservation. The viable UTF1-positive population of pre-pubertal stallion germ cells was also measured using immunocytochemistry after 1 and 3 months of cryopreservation. As expected, the viability, cell population, and viable cell population were significantly reduced after 1 and 3 months of cryopreservation. At the pre-pubertal stage, the addition of trehalose or PEG to 10% DMSO did not show any effect on the viability, cell population, viable cell population, or viable UTF1-positive germ cells at either 1 or 3 months after cryopreservation. However, at the post-pubertal stage, the viable population was significantly higher in germ cells that were cryopreserved with 5% or 10% PEG, than in the cells cryopreserved with 10% DMSO only. In conclusion, PEG at 5% or 10% added to 10% DMSO serves as an optimal cryoprotectant agent for the cryopreservation of germ cells from post-pubertal stallions.

1. Introduction

Spermatogonial stem cell (SSC) transplantation is considered an alternative method for maintaining male fertility (Avarbock et al., 1996). This technique can also be used to transmit genetic information from males to offspring over a long period. In mice, progeny were successfully produced using spermatozoa derived from frozen/thawed SSCs (Avarbock et al., 1996). Furthermore, fertile offspring have been produced in mice following transplantation of SSCs that had been cryopreserved for more than 14 years (Wu et al., 2012). The results of these studies indicate that SSCs can be cryopreserved for a long period without harmful effects on their function. Thus, the development of an optimal cryopreservation technique is essential for improving the success rate of offspring production by SSC transplantation after long-term preservation. In addition, a

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cryopreservation technique for stallion germ cells may contribute to investigating the biological activity of germ cells *in vitro* in a more efficient manner, in terms of conserving samples, than current methods. Successful cryopreservation of SSCs has been reported in several species including rhesus monkey (Hermann et al., 2007), goats (Kaul et al., 2010), boars (Lee et al., 2014), and bulls (Izadyar et al., 2002). However, the survival rate and proliferation capacity of spermatogonia vary greatly according to the type of cryoprotectant and freezing method used (Izadyar et al., 2002; Lee et al., 2014).

We previously reported the use of 10% dimethyl sulfoxide (DMSO) as a cryoprotectant to cryopreserve isolated germ cells from stallions (Jung and Yoon, 2016). However, the recovery rates of viable germ cell populations were not satisfactory; rates were approximately 32% and 17% in pre- and post-pubertal stallions, respectively. Thus, the main objective of the current study is to develop an optimal protocol for the cryopreservation of stallion germ cells. DMSO has been widely used as a cryoprotectant agent for several types of cells including germ cells. However, improvements in the viability and recovery rate of cells have also been reported with the addition of trehalose or PEG to the DMSO solution (Lee et al., 2013a,b, 2014; Kim et al., 2015). In the present study, we evaluated viability, cell population, viable cell populations, and viable UTF1-positive germ cell populations after cryopreservation using a 10% DMSO cryosolution with or without the addition of trehalose or PEG at different concentrations.

2. Materials and methods

2.1. Animals

Testes were obtained through a routine field castration service provided by veterinarians in the Republic of Korea. Testicular samples were obtained from pre-pubertal (1–1.5 yr, n = 6) and post-pubertal (3–7 yr, n = 5) stallions. The reproductive stages of the stallions were determined based on the morphological characteristics of the testes (Jung et al., 2014). The testes of pre-pubertal stallions were identified by the absence of a lumen opening as well as evidence of full spermatogenesis in a cross section of a seminiferous tubule (Jung et al., 2014).

2.2. Isolation of stallion germ cells

Single germ cells were isolated through a two-step enzyme protocol as previously described (Jung and Yoon, 2016). Testicular tissue (10 g) was removed from each testis and cut to a size of 1 cm^3. The first digestion was performed with collagenase type IV (1 mg/ml; Sigma, St. Louis, MO, USA) in Hanks' balanced salt solution (HBSS; Invitrogen, Carlsbad, CA, USA) for 10 min at 37 °C in a shaking incubator with vigorous shaking. Seminiferous tubules were pelleted using centrifugation at 200 × *g* (4 °C). The supernatant containing Leydig cells was removed, and a second digestion was performed using trypsin (2.0 mg/ml, Invitrogen) containing ethylenediaminetetraacetic acid (EDTA; 1.04 mM), and DNase I (1.4 mg/ml; Sigma) in HBSS for 15 min. Testicular cell solutions were quenched using fetal bovine serum (FBS, 10%) and filtered through a 70-μm cell strainer (SPL Lifesciences, Pocheon-City, Gyeonggi-do, Republic of Korea) to remove undigested Sertoli cells. After centrifugation at 600 × *g* for 10 min (4 °C), germ cell pellets were resuspended in minimum essential medium (MEM)α containing 10% FBS.

2.3. Cryopreservation of germ cells with different cryoprotectants

For cryopreservation, MEMα was mixed with 20% DMSO and 20% FBS; further, a cryoprotectant agent—either trehalose (99.0%, Sigma; 50, 100, or 200 mM) or PEG (800 mg/ml, Sigma; 2.5, 5, or 10%)—was added to the medium to form the cryosolution. Pelleted germ cells were reconstituted with MEMα at a concentration of 40×10^6 germ cells/ml and mixed with each type of cryosolution at a 1:2 dilution (50:50, final concentration at 20×10^6/ml) in a 1.8-ml cryovial (Corning, Midland, MI) (Hermann et al., 2007). After inverting, cryovials were placed in a Nalgene freezing container (Nalgene, Rochester, NY) filled with 250 ml Isopropyl Alcohol (DUKSAN Pure Chemicals Co., Ansan-si, Gyeonggi-do, Republic of Korea) and frozen overnight at −80 °C. Frozen samples were stored in liquid nitrogen for up to 3 months.

2.4. Immunocytochemistry

To evaluate the recovery rate of undifferentiated spermatogonia, immunocytochemistry was performed to identify germ cells expressing UTF1, a putative marker of undifferentiated equine spermatogonia (Jung et al., 2014). Frozen germ cells were thawed by immersing the conical tube in a 37 °C water bath for 1 min and then immediately resuspended in MEMα with 10% FBS to generate a single cell suspension. Germ cells (approximately 5×10^4 cells) were placed onto Fisherbrand™ Superfrost/Plus microscope slides (Fisher Scientific, Pittsburgh, PA, USA) and fixed with ice-cold methanol. Slides were air-dried overnight and washed twice with PBS for 5 min each. After blocking with donkey serum (blocking buffer) for 1 h at room temperature, slides were incubated with a rabbit UTF1 antibody (AB3383, Millipore, Billerica, MA, USA; 1:500) diluted in blocking buffer for 1 h 30 min at room temperature (20–25 °C). Slides were washed 3 times in PBST for 5 min each. Slides were incubated in donkey anti-rabbit IgG Alexa Fluor 488 secondary antibody (Life technologies, Grand Island, NY, USA; 1:1000) for 45 min at room temperature and washed 3 times with PBST for 5 min each. Mounting was performed with Vectashieldmounting medium containing 4,6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA, USA). UTF1 immunolabeling of germ cells was observed using a Leica DM 2500 microscope (Wetzlar, Germany). Images were captured using a Leica DFC 450C camera.

2.5. Assessing population, viability, and number of UTF1 positive germ cells

The total population of frozen/thawed germ cells and their viability were evaluated using a hemocytometer with