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Short communication

Open pulled straw vitrification of *in vitro* matured sheep oocytes using different cryoprotectants

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ABSTRACT

The aim of this study was to compare the survival and *in vitro* development of sheep oocytes after open pulled straw vitrification and different final concentrations of permeable cryoprotectants. In 5 identical replicates of two experiments, *in vitro* matured (IVM) oocytes were vitrified by the Open Pulled Straw (OPS) method, then warmed, and the surviving ones were subjected to parthenogenetic activation. In Experiment 1, survival rate of oocytes after vitrification in 33% ethylene glycol was higher than in 33% DMSO or a mixture of 17.5% ethylene glycol and 17.5% DMSO (87.64 vs. 77.43 vs. 69.39%, respectively). The cleavage and blastocyst rates were higher after vitrification in mixture group than in ethylene glycol and DMSO (46.81 and 15.5 vs. 37.55 and 9.12 vs. 29.51 and 6.40%, for cleavage and blastocyst rates in different groups, respectively). In Experiment 2, elevated concentrations of vitrification solutions were used. The survival rate was higher after vitrification in 40% ethylene glycol and in the mixture of 20% ethylene glycol and 20% DMSO than in 40% DMSO (90.22 vs. 87.56 vs. 75.34%, respectively). Cleavage and blastocyst rates were also higher in the ethylene glycol and ethylene glycol – DMSO mixture group than in DMSO alone group (50.67 and17.60 vs. 49.13 and 14.45 vs. 33.86 and 9.81% for cleavage and blastocyst rates in different groups, respectively). The survival rates between the two experimental groups was higher in 40% ethylene glycol group, 40% mixture group and 33% ethylene glycol group than in 40% DMSO group, 33% mixture group and 33% DMSO group. Cleavage and blastocyst rates were higher in 40% ethylene glycol group, 40% mixture group and 33% mixture group than in 40% DMSO group, 33% ethylene glycol group and 33% DMSO group. All cleavage and blastocyst rates in both the experiments were lower than those of the non-vitrified control group (87.00 and 45.00, respectively). In conclusion, although ethylene glycol group and ethylene glycol – DMSO mixture group gave better survival and cleavage – blastocyst rates than DMSO group, the survival rates were lower than the control group and hence the technique could be further improved to get better results after OPS vitrification of IVM sheep oocytes.

1. Introduction

Oocytes cryopreservation in mammalian species has gained a rapid pace during the past couple of decades emphasizing its importance in various assisted reproductive technologies. Cryopreservation of an oocyte is a

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challenging task because of its sensitive nature to chilling due to several factors, such as size, shape, permeability, species of origin, lipid content, that affect its survival and developmental competence after cryopreservation. Although recent approaches toward improving the technique have significantly increased the survival rates in some species, thorough investigation needs to be carried out to find a technique that could adjust to each species if required.

Slow freezing is the most widely used technique for ovine oocyte and embryo cryopreservation, but it requires an expensive biological programmable freezer and is time consuming (Dattena et al., 2004). The alternative to slow freezing is vitrification, which is being increasingly used as the emerging tool of choice for routine cryopreservation of oocytes and embryos (Baril et al., 2001). Vitrification is defined as a process by which a highly concentrated solution of cryoprotectant solidifies during cooling without formation of ice crystals (Rall, 1987). Oocyte vitrification has been developed using mouse embryos (Rall and Fahy, 1985). Vitrification has also been successfully attempted in cattle (Fuku et al., 1992; Vajta et al., 1997) goats (Begin et al., 2003).

The advantages of vitrification technology compared to slow-rate freezing are the low price of equipment, the simplicity of the procedure, and the short time required (Palasz and Mapletoft, 1996). One of the tools in vitrification is open pulled straw (OPS). Vitrification in OPS reduces the cryoprotectant volume and increases the freezing rate more than 10-folds when the straws are immersed in liquid nitrogen (Vajta et al., 1997; Lewis et al., 1999). Rapid cooling inhibits the formation of ice crystals and in addition toxic and osmotic effects at warming are minimized by immersion of the capillary containing the oocytes or embryos into a warming solution. Despite the convincing evidence regarding the superiority of vitrification for oocyte cryopreservation in several other species (Vajta and Kuwayama, 2006a; Vajta and Nagy, 2006b), there are few reports of vitrification of sheep oocytes (Dike, 2009; Martinez and Matkovic, 1998; Asgari et al., 2012).

The objective of this study was to evaluate the post-thaw survival of *in vitro* matured abattoir derived sheep oocytes during vitrification using different concentrations of permeable cryoprotectants, ethylene glycol (EG) and dimethyl sulphoxide (DMSO).

2. Materials and methods

All chemicals and media were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Disposable plastic-wares were obtained from Nunc (Roskilde, Denmark) unless otherwise mentioned.

2.1. In vitro maturation of oocytes

Abattoir derived ovaries from mixed breeds of sheep approximately above three years of age were collected and washed three times with warm isotonic saline (32–37 °C) containing 400 IU mL^{-1} penicillin and 500 μg mL^{-1} streptomycin and transported to the laboratory within 2–4 h. Oocytes from follicles (2–8 mm in diameter) were harvested by puncturing with an 18-gauge needle. The collection medium consisted of Hepes buffered Tissue Culture Medium-199 (M-199) containing 0.3% bovine serum albumin (BSA). The oocytes were washed two to four times with the washing medium which consisted of Hepes buffered M-199 supplemented with 10% fetal bovine serum (FBS) (Hyclone, Canada), 0.68 mM L-glutamine, 0.8 mM sodium pyruvate and 50 μg mL^{-1} gentamicin. Cumulus–oocyte complexes (COCs) having a compact and unexpanded cumulus mass with equal to or greater than three layers of cumulus cells and homogenous granular ooplasm were used for *in vitro* maturation (IVM). After washing thrice with IVM medium (bicarbonate buffered M-199 supplemented with 10% FBS, 5 μg mL^{-1} pFSH, 1 μg mL^{-1} estradiol- 17β, 0.8 mM sodium pyruvate and 50 μg mL^{-1} gentamicin), groups of 15–20 COCs were cultured in 100-μL droplets of IVM medium, overlaid with sterile mineral oil in 35-mm diameter Petri dishes, and cultured for 24 h in 5% CO_2 with maximum humidity at 38.5 °C (Fig. 1).

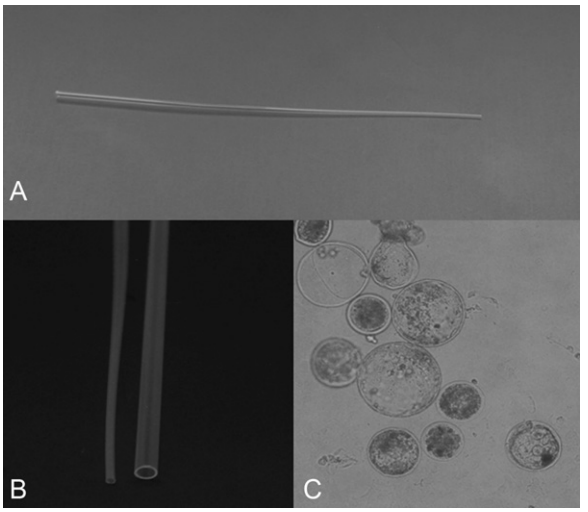

Fig. 1. Open pulled straw (A); comparative thickness of OPS and conventional straw (B); blastocysts after *in vitro* culture of vitrified IVM oocytes (C).

2.2. Vitrification

COC'S after IVM, having expanded cumulus, were stripped of their cumulus using hyaluronidase (0.5 mg mL^{-1}) in T2 (where T denotes Hepes buffered M-199 supplemented with 2.0 mM L-glutamine, 0.2 mM sodium pyruvate, 50 μg mL^{-1} gentamicin and the following number denotes the percentage of FBS, here 2%). All the denuded oocytes with compact and regular cytoplasm were used for vitrification. Vitrification by OPS method was conducted as described by Vajta et al. (1997). The vitrification solutions were prepared in a 4-well dish, the well 1 and well 2 of the dish contained T20, the well 3 contained the equilibration medium (20% or 33% cryoprotectant in T20) and the well 4 contained the vitrification medium (33% or 40% cryoprotectant) in T20 containing 1 M sucrose. Oocytes were equilibrated for 5 min in a holding medium in well 2 of the 4 well dish, transferred for 5 min to the equilibration medium (T20 with either 15% or 20% of EG or DMSO or both EG and DMSO), followed by 20 s in the vitrification medium (T20 with either 33% or 40% EG or DMSO or both EG and DMSO supplemented with 1 M sucrose). Oocytes were loaded in the OPS via capillarity action, by placing the narrow end of the pulled straw into the holding medium. The loading volume was approximately 2 μL. The straws were then immediately submerged into liquid nitrogen.

2.3. Warming

Warming was performed by using serial dilution (Attanasio et al., 2010) method. Briefly, the end of the straw was directly immersed in a holding medium containing 1 M sucrose solution in T20 for 1 min and then transferred into 0.5 M sucrose and were left for 5 min in this medium and then transferred for 5 min in a medium containing 0.25 M sucrose and finally into holding medium without sucrose. All equilibration and dilution steps as well as warming were performed at room temperature (approximately 25 °C). The oocytes with dense and regular cytoplasm with intact cell membrane and zona pellucida after warming were considered as normal oocytes and were used for parthenogenetic activation.