Short communication

Effect of cryotop vitrification on preimplantation developmental competence of murine morula and blastocyst stage embryos

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Abstract

Vitrification is an effective method for the cryopreservation of mammalian embryos. Nevertheless, it is unclear which embryonic developmental stage is the most suited for vitrification and would ensure maximal developmental competence upon subsequent warming. This study, therefore, compared the effects of cryotop vitrification on the developmental competence of murine morula and blastocyst stage embryos. Additionally, trophectoderm (TE) and inner cell mass (ICM) cell numbers were compared in two hatched blastocyst groups derived from vitrified morulae and blastocysts, respectively. The post-vitrification survival rates for mouse embryos at the morula and blastocyst stage were 95.4% (186/195) and 96.5% (195/202), respectively. The blastocyst formation rate was significantly lower for vitrified morulae (90.3%) compared with the non-vitrified control group (98.4%) ($P < 0.05$). The hatching rates were similar between the vitrified morula (79.6%) and the vitrified blastocyst (81.0%) groups. When further development to the fully hatched blastocyst stage was compared, fully hatched blastocysts derived from vitrified morulae had significantly higher cell counts for both the ICM and TE lineage, as compared with hatched blastocysts derived from vitrified blastocysts ($P < 0.001$). Cryotop vitrification of mouse embryos at the morula stage rather than blastocyst stage would thus ensure a higher degree of post-warming developmental competence.

Keywords: *blastocyst, development, morula, preimplantation, vitrification*

Introduction

To date, vitrification, which was first described by Rall and Fahy (1985), is an inexpensive and widely used method for the cryopreservation of mammalian embryos. In order for vitrification to take place, there must be high concentrations of cryoprotectants and solutes together with a rapid decrease in temperature. Because ice crystallization is avoided, this minimizes damage to mammalian embryos during cryopreservation. In the last two decades, there were many successful studies on the vitrification of mammalian embryos with various different carriers including open-pulled straws (Kuleshova *et al.*, 1999; Chen *et al.*, 2000; Cuello *et al.*, 2008), electron microscopy copper-grid (Park *et al.*, 1999; Son *et al.*, 2003), hemistraw system (Vandervost *et al.*, 2001; Liebermann and Tucker, 2002), nylon mesh (Matsumoto *et al.*, 2001), cryoloop (Lane *et al.*, 1999; Mukaida *et al.*, 2001, 2003) and cryotop (Hiraoka *et al.*,

2004; Hochi *et al.*, 2004; Kuwayama *et al.*, 2005). It must be noted that these various carriers yielded different post-warming recovery rates after vitrification.

Another concern is the optimal embryonic developmental stage best-suited for vitrification, which would ensure the highest developmental competence after warming. Mammalian preimplantation development spans the cleavage stages (2-cell to 8-cell stage), compaction and morula formation, followed by cavitation with the formation of a blastocyst, and subsequent hatching of the fully expanded blastocyst from its zona pellucida. The embryonic genome begins to be transcribed from the 2-cell stage in mice (Evsikov *et al.*, 2004) and the 4- to 8-cell stage in humans (Braude *et al.*, 1988). When the embryo has around 32 cells, a fluid-filled cavity (blastocoele) appears, leading to the formation of a blastocyst. Blastocyst formation marks the differentiation of two distinct embryonic lineages: the epithelial trophectoderm (TE), which is specialized for implantation and the pluripotent inner cell mass (ICM), from which the fetus is derived (Wang and Dey, 2006).

Theoretically, vitrification could be applied to embryos at all preimplantation stages. However, it is yet unknown which particular embryonic developmental stage would tolerate the vitrification process better and yield the highest survival rate and best developmental competence after warming. It has been demonstrated in the porcine model that vitrification is best-suited for blastocyst stage embryos using the cryoloop as carrier (Nawroth *et al.*, 2005). Additionally, it has also been reported that cryo-tolerance of porcine embryos is highest at the blastocyst stage, particularly at the early blastocyst stage (Ushijima et al., 2004).

Nevertheless, cryopreservation at the morula stage may theoretically be better than cryopreservation at the blastocyst stage, as this can avoid the detrimental effects of prolonged in vitro culture (still inferior compared with the physiological in vivo environment), as well as avoid the problems associated with excess blastocoelic fluid. A few studies showed that the developmental competence of vitrified blastocysts by open-pulled straw was comparable to that of fresh embryos, which would imply that the early blastocyst stage is the most optimal stage for mouse embryo cryopreservation (Cuello *et al.*, 2004; Zhou *et al.*, 2005). However, other studies had contrary results which showed that the survival and blastocyst hatching rates of vitrified morulae were, in fact, higher than those of vitrified early blastocysts, when cryostraws were utilized ($P < 0.05$). Morula is likely the most optimal stage for embryo vitrification (Han *et al.*, 2003; Zhao *et al.*, 2004). Recently, cryotop vitrification is being used to cryopreserve human embryos (Hiraoka *et al.*, 2007). Hochi *et al.* (2004) utilized Cryotop instead of GL-tip or Cryoloop for vitrification of rabbit zygotes without changing other conditions, and observed better embryonic development. However, it is unknown which embryonic developmental stage would yield the best developmental competence upon vitrification.

This study compared the effects of vitrification on the developmental potential of murine morula and blastocyst stage embryos utilizing the cryotop system as carrier. After warming, in vitro culture was used to assess embryo morphology and the ability of an embryo to leave the zone pellucida (hatching). Furthermore, the cell number counts were determined for the ICM and TE of fully hatched blastocysts derived from vitrified morulae and blastocysts, as an additional indicator of blastocyst quality.

Materials and methods

Ethical approval for scientific research

All experiments involving the use of animals were in accordance with the Council for International Organizations of Medical Sciences (1985), and the experimental protocol was approved by the Institutional Animal Care and Use Committee of Nanjing Medical University.

Embryo collection

Female ICR mice, 6–8 weeks old, were induced to superovulate with an injection of 10 IU of pregnant mare's serum gonadotrophin (cat. no. CSG600, Sansheng Pharmaceuticals, Ningbo, China) followed with 10 IU of human chorionic gonadotrophin (Pregnyl^{TM}; Livzon Syntpharm, Zhuhai, China) administered 48 h later. Females were mated with males from the same strain and inspected for the presence of vaginal plugs on the following morning. Females with the presence of vaginal plugs were considered to be pregnant and were killed 46–48 h post human chorionic gonadotrophin by cervical dislocation. Morula-stage embryos were flushed from the dissected oviducts with morpholino propane sulphonic acid (MOPS)-buffered G1 medium (G-MOPS^{TM}; Vitrolife, Gothenburg, Sweden). Morphologically normal morulae were selected, washed and transferred to G1^{TM} media (Vitrolife) containing 10% synthetic serum supplement for continuous culture at 37°C in a humidified atmosphere of 6% CO_2 in air. Embryos were cultured in groups of 20 in 20 µl of culture medium under paraffin oil (G-OIL^{TM}; Vitrolife) for 1 h prior to the vitrification study. Morulae were allocated to either the vitrification or control group. In a parallel experiment, day-3.5 blastocysts were collected and vitrified using the same procedure. All cultures were performed at 37°C in a humidifed atmosphere of 6% CO_2 in air. The warmed morulae were cultured for 24 h to the blastocyst stage, and for 48 h for hatched blastocyst formation. Warmed blastocysts were cultured for 24 h for hatched blastocyst formation.

Vitrification and warming

Embryos were vitrified using the cryotop as carrier, as described by Vandervost *et al.* (2001) and Liebermann and Tucker (2002). Briefly, one end of a 0.25 ml sperm-freezing straw (CryoBioSystem straws; IMV Technologies, L'Aigle Cedex, France) was cut at a slant angle with a sharp scalpel, so that the inner surface of the straw was exposed (about 1 cm in length). This made it easy to pipette a small droplet of liquid (<1.0 µl) onto the open inner surface of the straw. Vitrification was then carried out with the use of the Kitazato Vitrification Kit (Kitazato BioPharmaceuticals,