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Supplement of tauroursodeoxycholic acid in vitrification solution improves the development of mouse embryos

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Abstract

This study was performed to determine whether supplementation of tauroursodeoxycholic acid (TUDCA), an endoplasmic reticulum (ER) stress inhibitor, during vitrified cryopreservation enhances the development of frozen mouse embryos. Mouse 8-cell stage embryos were collected and exposed to a cryoprotectant solution containing TUDCA or TM (tunicamycin, an ER stress inhibitor) at room temperature and stored in liquid nitrogen following vitrification. The final concentration of TUDCA or TM was 50 μM. The survival and development rates of mouse 8-cell stage embryos exposed to TUDCA- or TM-containing solutions at room temperature or stored in liquid nitrogen following vitrification were measured. There were no significant differences in survival rate and blastocyst formation rate among control, TUDCA, and TM groups after embryos were exposed to vitrification solutions at RT. When mouse 8-cell stage embryos were treated with TUDCA or TM and then stored in liquid nitrogen, the survival rates of control and TUDCA groups were significantly higher than for the TM group. Blastocyst formation rate of the TUDCA group following *in vitro* culture was significantly higher than that in control or TM groups. The TM group showed a lower ($p < 0.05$) blastocyst formation rate than the other two groups. Our results indicate that TUDCA supplementation during cryopreservation of mouse embryos could enhance their development capacity.

Keywords: mouse embryo, TM, TUDCA, vitrification

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Introduction

The endoplasmic reticulum (ER) is a principal site for the processing of transmembrane proteins and lipids in the cell and for maintaining intracellular calcium homeostasis (Groenendyk and Michalak, 2005). Stress conditions trigger the accumulation of unfolded or misfolded proteins in the ER by activation of the unfolded protein response in cells (Ron and Walter, 2007). When homeostasis in the ER is perturbed or when ER protein folding capacity is overloaded, the prolonged stress will result in a cell fate of apoptosis. A short-term ER stress response can protect cells, but long-term or severe ER stress induces cell apoptosis.

ER stress plays an important role in mammalian oocyte maturation and embryo development (Latham, 2015). Previous studies showed that cryopreservation not only caused ultrastructural injuries

and apoptosis in human oocytes (Gualtieri et al., 2009), but also induced ER stress in mice (Zhao et al., 2015). Tauroursodeoxycholic acid (TUDCA), which is known to be an ER stress inhibiter, has been reported to combat ER stress during embryo development in mice (Zhang et al., 2012a; Lin et al., 2015), pigs (Kim et al., 2012; Zhang et al., 2012b), cattle, and buffalo (Song et al., 2011; Sharma et al., 2015). However, TM (Tunicamycin, an ER stress inducer) had a negative effect on embryonic development due primarily to elevated ER stress (Zhang et al., 2012b; Sharma et al., 2015). To the best of our knowledge, although the effects of TUDCA or TM on embryo development have been extensively studied, very few studies report the effects of supplementation during cryopreservation on subsequent embryo development and cryotolerance in mammalian embryos. As such, the aim of this study was to test the effect of TUDCA or TM supplementation with cryoprotectants in vitrification of mouse 8-cell stage embryos on survival and development rates.

Materials and Methods

Mouse 8-cell stage embryo collection

All animal experiments were approved by the Institutional Animal Care and Use Committee of Chungnam National University (CNU-00373). Superovulation of female B6D2F1 mice (4 - 6 week old) was induced by injecting 5 IU PMSG (G4877, Sigma-Aldrich, St. Louis, Mo, USA) intraperitoneally followed, 48 h later, by injection of 5 IU hCG (C1063, Sigma-Aldrich, St. Louis, Mo, USA). Then, females were mated with B6D2F1 male. The next morning, females with vaginal plugs were isolated and used for the experiment. The day on which a vaginal plug was found was designated 0.5 day *post coitum* (0.5 dpc). Mouse 8-cell embryos were collected by flushing oviducts using a manually pulled glass Pasteur pipette at 2.5 dpc.

Vitrification solutions

The solution preparation vitrification, and thawing were performed as described in previous reports (Gibbons et al., 2011; Maehara et al., 2012; Moawad et al., 2013) with some modifications. The solutions were as follows; basal solution (BS): M2 medium (M7167, Sigma) + 20% FBS (fetal bovine serum, Gibco), equilibration solution (ES): BS + 7.5% DMSO (D2650, Sigma) + 7.5% EG (ethylene glycol, 324558, Sigma), vitrification solution (VS): BS + 15% DMSO + 15% EG, thawing solution (TS): TS1: BS + 1 M sucrose (S0389, Sigma); TS0.5: BS + 0.5 M sucrose. TUDCA (T0266, Sigma) or TM (T7765, Sigma) were added in BS medium at a final concentration of 50 μM or 1 μg/mL.

Toxicity test of vitrification solutions

To test the toxicity of vitrification solutions, mouse 8-cell stage embryos were exposed to a series of different solutions at room temperature (RT). In brief, mouse 8-cell embryos were washed in BS and then placed in ES for 3 min for equilibration. Embryos were then transferred into VS and, after 30 s incubation in VS, they were transferred into fresh TS1 and TS0.5 mediums for 3 min, respectively. After a 3 min treatment with BS, the embryos were washed with M16 and then cultured in M16 medium *in vitro*. The survival and development ability of mouse 8-cell stage embryos were evaluated after incubation for 1 h and 48 h, respectively.

Embryo vitrification and thawing procedures

Mouse 8-cell stage embryos were washed in BS and then transferred into ES for 3 min. After 3 min equilibration, embryos were placed in 4 mL VS in a 35 mm plastic dish for 30 s and then embryos were transferred into 2 μL fresh VS drops. Embryos (about five 8-cell stage embryos) in 2 μL VS were aspirated into a small plastic tipped micropipette by siphoning and loaded into cryo-tubes (1.8 mL, Thermo). Subsequently, embryos were stored in liquid nitrogen for at least 1 week. For thawing, cryo-tubes were kept at 37℃ for 30 s. Embryos were immediately transferred into 2 mL TS1 medium and kept warm for 3 min. Embryos were placed in 2 mL TS0.5 medium for 3 min and then to BS for 3 min. All thawing procedures were performed at 37℃.

Assessment of survival and development ability of vitrified embryos

After thawing, embryos were washed with M16 and cultured with the same medium in an incubator at 37℃, 5% CO_2 in air. After 1 h of incubation, survival of vitrified mouse 8-cell stage embryos was assessed by morphological evaluation (Fig. 1) under a stereomicroscope (Nikon, Japan) with an ocular scale at a magnification of 50x. To examine the developmental potential, mouse embryos continued to be cultured *in vitro* in M16 medium for 48 h after which development of vitrified 8-cell embryos to the blastocyst stage was assessed.

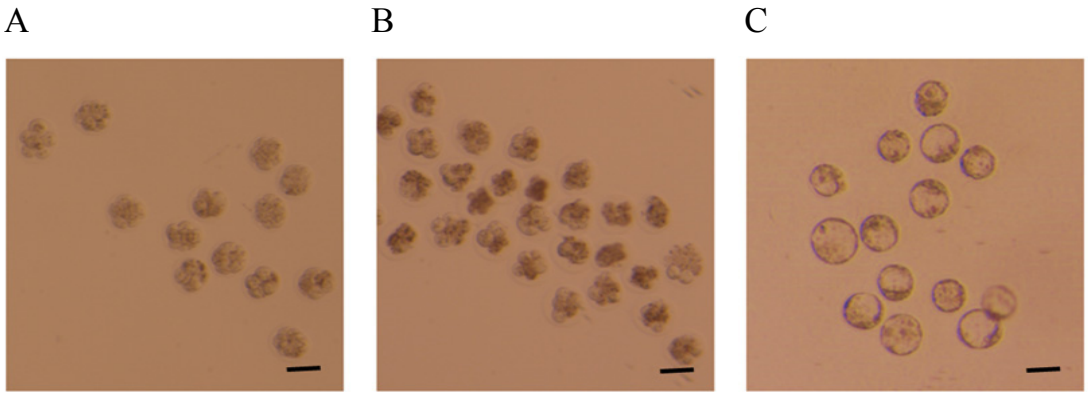

Fig. 1. Morphology of mouse embryos in vitrification process. A: Normal thawed mouse embryos with normal blastomeres and intact zona pellucida after vitrification. B: Abnormal thawed embryos with lysis of blastomeres or broken zona pellucida. C: Blastocysts derived from vitrified 8-cell stage embryo following in vitro culture. Scale bar represents 100 μm.

Statistical analysis

Percentage data of survival and blastocyst formation rates were subjected to arcsine transformation before analysis, and then analyzed by one-way ANOVA and Fisher's protected least significant difference (LSD) test using SPSS 17.0 (SPSS Inc., Chicago, IL, USA) software. The results are expressed as means ± SE. Data were considered to be significantly different when p values were less than 0.05.

Results

Survival and blastocyst formation rates of embryos exposed to different vitrification solutions at room temperature

First, the toxicity of vitrification solutions containing TUDCA or TM on mouse embryos were tested following exposure at RT. As shown in Table 1, there were no significant differences in the survival rates (97.7 ± 1.4, 97.8 ± 1.3, 95.8 ± 1.5%; control, TUDCA, TM groups, respectively) and blastocyst formation rates (81.1 ± 2.4, 82.3 ± 1.4, 76.2 ± 1.4%; control, TUDCA, TM groups, respectively) among three treatment groups. However, the TM group showed lower survival and blastocyst formation rates than the control and TUDCA groups.

Table 1. The survival rate (%) and blastocyst formation rate (%) of mouse 8-cell stage embryos exposed to vitrification solution containing TUDCA or TM at RT.

	N	Survived (%)[y]	Blastocyst (%)[z]
Control	84	97.7 ± 1.4	81.1 ± 2.4
TUDCA	87	97.8 ± 1.3	82.3 ± 1.4
TM	89	95.8 ± 1.5	76.2 ± 1.4

[y,z]The survival rate is given as the percentage of survived embryos among total embryos, while the blastocyst rate reflects the percentage of blastocysts in total survived embryos.

Survival and development rates of embryos after vitrification

In the second experiment, the survival and development rates of mouse 8-cell stage embryos after vitrification were estimated. As shown in Table 2, the survival rates in control and TUDCA groups (76.5 ± 2.4% and 79.7 ± 1.7%, respectively) are significantly higher than those in the TM group (69.6 ± 2.3%). The blastocyst formation rate in the TUDCA group (48.3 ± 5.1%) is significantly higher than rates of the control and TM groups (43.7 ± 1.1% and 26.0 ± 1.2%, respectively). However, the TM group showed lower ($p < 0.05$) blastocyst formation rates than TUDCA and control groups.

Table 2. The survival rate (%) and blastocyst formation rate (%) of mouse 8-cell stage embryos after vitrification.

	N	Survived (%)[y]	Blastocyst (%)[z]
Control	93	76.5 ± 2.4a	43.7 ± 1.1b
TUDCA	99	79.7 ± 1.7a	48.3 ± 5.1a
TM	99	69.6 ± 2.3b	26.0 ± 1.2c

Different letters within a column indicate significant differences ($p < 0.05$).
[y,z]The survival rate is given as the percentage of survived embryos among total embryos, while the blastocyst rate reflects the percentage of blastocysts in total survived embryos.

Discussion

Cryopreservation of embryos or oocytes is considered to be an effective method for preserving fertility in assisted reproductive technologies (ART). Despite ongoing advances in cryopreservation technology, the overall efficiency of

embryo cryopreservation remains low (Saragusty and Arav 2011; Moawad et al., 2013). In the current study, we tested the effect of TUDCA supplementation during vitrification on the survival and development rates of mouse embryos. Our results suggest that a supplement of TUDCA during vitrification improves blastocyst formation rates of vitrified mouse 8-cell stage embryos by attenuating ER stress.

The beneficial influence of TUDCA supplement on *in vitro* culture (IVC) of embryos has been reported in many mammalian species. In pigs, supplements of 50 or 200 μM TUDCA significantly increased blastocyst formation rates and reduced apoptosis in parthenogenetic (Zhang et al., 2012b) or *in vitro* fertilized embryos (Kim et al., 2012). In buffalo, supplement of 50 μM TUDCA during IVC significantly reduced endoplasmic reticulum stress (Sharma et al., 2015). In mice, supplement of 50 μM TUDCA during IVC significantly improved preimplantation embryonic development, implantation, and live-birth rates (Zhang et al., 2012a; Lin et al., 2015). In the current study, we investigated the effect of TUDCA or TM supplementation during vitrification on the survival and development rates of mouse 8-cell stage embryos. TUDCA or TM were used to attenuate or induce ER stress during vitrification procedures. Our results indicated that TUDCA supplementation during vitrification enhanced the rate of blastocyst formation. However, TM decreased the survival and development rates of vitrified mouse embryos, probably due to induced ER stress. The vitrification procedure itself could cause an extra ER stress to embryos. Our result is consistent with prior studies that have reported that TUDCA improved the survival and blastocyst formation rates of vitrified mouse metaphase II stage oocytes and their subsequent embryonic development competence (Zhao et al., 2015).

We also investigated the toxicity of vitrification solutions, but the TUDCA treatment did not improve the blastocyst formation rate compared to control. This result indicates that exposure to vitrification solutions does not produce an extra ER stress effect in embryos during exposure to vitrification solutions in freezing process. Although there were no significant differences in the rates of survival and blastocyst formation among control, TUDCA- and TM-treated groups, blastocyst formation rate in TM treatment group decreased when compared with TUDCA and control groups. This result indicates that extra ER stress induced during the vitrification process had a negative effect on mouse embryos, even though embryos were not subjected to vitrification.

Taken together, the cryopreservation medium supplemented with TUDCA effectively improves mouse embryo development into the blastocyst stage by reducing ER stress. In addition, TM supplementation could affect developmental potential of mouse embryos during the vitrification process. TUDCA treatment could be capable of lessening ER stress during development of vitrified embryos and enhance the viability and quality of blastocysts.

Conclusion

The results of this study demonstrated that supplementation of vitrification solutions with TUDCA improves the development rate of mouse 8-cell stage embryos by attenuating ER stress. However, TM treatment reduced the rates of survival and development of mouse 8-cell stage embryos after thawing vitrified embryos.

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