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# EFFECT OF TREHALOSE DILUTION ON THE SURVIVAL OF VITRIFIED-THAWED MOUSE MORULAE

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Key words: Trehalose, mouse morulae, vitrification, dilution

The successful cryopreservation of mouse embryos by vitrification was first reported by RALL and FAHY[15]. This new technique involves the use of highly concentrated solutions of cryoprotectants which supercool and solidify into a glass during cooling at subzero temperature without the formation of intra- and extracellular ice.

One of the important factors that influences the viability of vitrified or frozen embryos is the method used for cryoprotectant dilution after thawing[14,17]. Hence, an efficient post-thaw dilution procedure to remove intracellular cryoprotectants from vitrified or frozen embryos is necessary to minimize osmotic stress and achieve high embryo survival.

Cryoprotectant dilution is usually carried out either by the slow stepwise method or the sucrose dilution method[10,17]. The use of non-permeable sugars like sucrose for cryoprotectant dilution has been reported by many investigators[2,3,6,8–13,18]. Sucrose functions as an osmotic buffer to restrict water movement across the membranes, and thus control the amount of swelling during dilution. Trehalose[4], a non-reducing disaccharide of glucose, which has been suggested to maintain membrane integrity during dehydration, has also been successfully used for dilution after thawing of rapidly frozen[7] and conventionally frozen mouse embryos[5]. However, to our knowledge there has been no report on the use of trehalose to dilute cryoprotectants from vitrified-thawed embryos.

This study was designed to determine if trehalose is effective for cryoprotectant dilution of vitrified embryos after thawing, and whether the length of exposure to trehalose during dilution affects embryo survival.

Four- to 7-week-old female ICR mice were superovulated by intraperitoneal injection of 5 IU of pregnant mare serum gonadotrophin (Serotrophin; Teikoku Zoki, Japan) followed 48 hr later by 5 IU of human chorionic gonadotrophin (hCG; Gonatrophin; Teikoku Zoki, Japan). These mice were mated singly with males of the same

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strain and then inspected the following morning for the presence of vaginal plugs (Day 1).

Compacted morulae were flushed from the excised oviducts and uteri at 76 to 78 hr after hCG injection with Dulbecco's phosphate-buffered saline plus 10% calf serum (PBS+CS). The collected embryos were washed three times in PBS+CS, pooled and held at room temperature until they were vitrified or directly cultured in vitro (control).

The vitrification solutions developed by Scheffen *et al*[16] were used in this study. The first solution (VS1) was composed of 10% glycerol+20% propylene glycol (v/v), while the second solution (VS2) was composed of 25% glycerol+25% propylene glycol (v/v). Both solutions were prepared in PBS+CS.

Eight to 12 embryos were equilibrated in VS1 at room temperature (20°C) for 10 min. The embryos were then pipetted and transferred to 20 $\mu$l of VS2 in a 0.25 ml French straw (IMV, L'Aigle, France). The column of VS2 in the straw was separated by two air bubbles from the 1 M trehalose (Sigma, St Louis, Mo., USA) in PBS+CS placed on each side (200 $\mu$l). Within 30 to 40 sec after loading and sealing the end of the straw, the straw was held vertically and plunged directly into liquid nitrogen ($LN_2$). The vitrified embryos were stored for 1 to 23 days in $LN_2$ until thawing.

Thawing was carried out by gently shaking the straw in a 20°C water bath until the ice had disappeared from the trehalose solution (about 15 sec). Immediately after thawing, the contents of each straw were expelled into a plastic culture dish and held for 5, 10, 20, 40 or 60 min at room temperature. The embryos were then collected and washed three times in PBS+CS.

All recovered embryos were cultured in microdrops of Whitten's medium (1969)[1] under paraffin oil at 37°C in 5% $CO_2$ in air. Nonvitrified fresh embryos (control) were also cultured in vitro using the same medium. Embryo viability was assessed by the development of the vitrified-thawed embryos to the expanded blastocyst stage after 48 hr of in vitro culture[16,19]. The viability results were analyzed by the Chi-square test.

The results of this study are shown in the Table. High survival rates were obtained when the vitrified embryos were diluted with trehalose for 5, 10, 20 and 40 min after thawing (89.4, 91.7, 87.2, and 88.6%, respectively), but survival rates significantly decreased ($P<0.05$) when the vitrified embryos were diluted in trehalose for 60 min after thawing (75.9%), as compared to the control (97.4%). It was further observed that there was a significant difference between exposure times of 10 and 60 min ($P<0.05$). However, no significant difference was observed between any other exposure times.

The present study indicates that trehalose can be effectively used to dilute the embryos out of the highly concentrated vitrification solution after thawing. The high (75.9–91.7%) survival rates obtained in this study are similar to those obtained with sucrose-diluted vitrified-thawed embryos[16,19]. Our results also agree with the report

Table. Survival rates of vitrified-thawed embryos after trehalose dilution at various exposure times.

| Exposure time to Trehalose-VS2 (min) | Development to expanded blastocyst stage after in vitro culture (%) |
|---|---|
| 5 | 42 / 47 (89.4) [ab] |
| 10 | 44 / 48 (91.7) [a] |
| 20 | 41 / 47 (87.2) [ab] |
| 40 | 39 / 44 (88.6) [ab] |
| 60 | 41 / 54 (75.9) [b] |
| Control* | 37 / 38 (97.4) [a] |

Viability = No. embryos viable / no. embryos recovered; each group had 4 to 5 replicates; 8-12 embryos / treatment

* Represents the percentage in vitro viability of embryos neither vitrified nor exposed to Trehalose-VS2 mixture

a, b : Values with different superscripts are significantly different ($P<0.05$).

of HONADEL and KILLIAN[5] who achieved high viability with the use of trehalose for 1-step dilution of the cryoprotectants from conventionally frozen-thawed mouse embryos.

The results of this study further show that increasing the exposure time to trehalose up to 40 min during dilution does not have a detrimental effect on embryo viability. This finding indicates that trehalose can be used for 1-step in straw dilution of the vitrification solution, and may permit the direct transfer of vitrified-thawed embryos to the recipient.

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