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Research Article

Effect of Three Different Cryoprotectant Solutions in Solid Surface Vitrification (SSV) Techniques on the Development Rate of Vitrified Pronuclear-Stage Mouse Embryos

Haydar BAĞIŞ*, Hande ODAMAN MERCAN
TÜBİTAK, Research Institute for Genetic Engineering and Biotechnology (RIGEB), P.O. Box 21, 41470 Gebze, Kocaeli - TURKEY
Yakup KUMTEPE
Department of Obstetrics and Gynecology, Faculty of Medicine, Atatürk University, Erzurum - TURKEY

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Abstract: The objective of this study was to compare the effects of three different vitrification solutions on the development of pronuclear-stage (PN) Dinnyes mouse embryos into blastocyst stage after vitrification by solid surface vitrification (SSV) technique. For this aim, it was compared three experimental groups and a control group. Experimental groups were distinguished each other by the vitrification solution used in SSV. It was used 4 % Ethylene Glycol (EG) at 37 ºC equilibration temperature in the first group (SSV-EG), 4% Dimethyl Sulfoxide (DMSO) at room temperature in the second group (SSV-DMSO) and 4% Propylene Glycol (PG) at room temperature in the third group (SSV-PG). After vitrification, the survived embryos were cultured to blastocyst stage in KSOM. It was determined a significant difference between the groups of SSV-EG and SSV-PG at developing rate to 2-cell stage ($P < 0.05$). Similarly, SSV-PG demonstrated significant differences with SSV-DMSO and the control group at developing rate to 3-8-cell stage ($P < 0.05$). When compared the rates of developing to morula stage among the groups, it was determined significant differences between SSV-PG and the control group at ($P < 0.05$); and between SSV-DMSO and SSV-PG ($P < 0.01$). Finally, it was compared the developing rates into blastocyst stage and found that SSV-EG demonstrated significant differences with SSV-PG and SSV-DMSO ($P < 0.01$). This study has shown that EG, DMSO and PG with trehalose can be used effectively as a cryoprotective agent in the quick freezing of pronuclear-stage mouse embryos. Finally, additional studies are needed to optimize the SSV method in further stage mouse embryos.

Key Words: Vitrification, pronuclear-stage mouse embryos, ethylene glycol, dimethyl sulfoxide, propilen glycol, trehalose

SSV Tekniği ile Dondurulan Pronükleer Fare Embriyolarının Dondurulmasında Üç Farklı Kriyoprotektan Maddenin Etkileri

Özet: Çalışmanın amacı, fare zigotlarının, Katı Yüzey Vitrifikasyonu (Solid Surface Vitrifikasyon; SSV) tekniği ile vitrifikasyon işleminin ardından blastosist safhasına dek gelişimleri üzerine, üç farklı vitrifikasyon solusyonunun etkilerini karşılaştırmaktır. Bu amaçla, üç deney ve bir kontrol grubu oluşturulmuştur. Deney grupları, SSV tekniği kapsamında farklı vitrifikasyon solusyonlarının kullanılması ile birbirlerinden ayrılmaktadır. Birinci grupta % 4 EG ve 37 ºC ekilibrasyon ısısı (SSV-EG); ikinci grupta % 4 DMSO ve oda ısısında ekilibrasyon (SSV-DMSO); son grupta ise oda ısısında ekilibrasyon ve %4 PG (SSV-PG) parametreleri kullanılmıştır. Virifikasyon işlemi sonrasında canlı kalan embriyolar KSOM medyumu içinde blastosist safhasına dek kültüre edilmiştir. SSV-EG ve SSV-PG grupları arasında 2-hücreli safhaya gelişim oranlarında istatistiksel olarak önemli bir fark saptanmıştır ($P < 0,05$). Benzer olarak, SSV-PG ve SSV-DMSO grupları arasında da 3-8-hücreli safhaya gelişim oranlarında istatistiksel olarak önemli bir fark saptanmıştır ($P < 0,05$). Gruplar arasında morula safhasına gelişim oranları karşılaştırıldığında, SSV-PG ve kontrol grubu arasında ($P < 0,05$) ve SSV-DMSO ile SSV-PG grupları arasında ($P < 0,01$) istatistiksel olarak önemli bir fark saptanmıştır. Sonuç olarak, blastosist safhasına gelişim oranları incelendiğinde, SSV-EG grubunun SSV-PG ve SSV-DMSO grupları ile önemli derecede farklılık gösterdiği belirlenmiştir ($P < 0,01$). Bu çalışma, EG, DMSO ve PG'nin, tek hücreli fare embriyolarının hızlı dondurulmasında kriyoprotektan ajan olarak etkin bir şekilde kullanılabileceğini göstermiştir. Özetle; gelişimin daha ileri safhalarındaki fare embriyolarının dondurulması protokollerini optimize etmek için ek bazı deneysel çalışmalara ihtiyaç duyulmaktadır.

Anahtar Sözcükler: Vitrifikasyon, pronükleer safhada fare embriyosu, etilen glikol, dimetil sülfoksit, propilen glikol, trehaloz

e-mail: haydar@rigeb.gov.tr

Introduction

The discovery of cryoprotective activity of glycerol for semen have given scientists opportunity to perform research on cryopreservation embryos. The first succesful offspring frozen-thawed embryos were reported in mice and later in other laboratory animals in 1972 (1). The generation of transgenic animals is equally important for basic science and medical, pharmaceutical and agricultural applications. DNA microinjection into pronuclear-stage (PN) embryos is the most frequently used method for the production of transgenic animals (2-4). An efficient pronuclear microinjection system requires the collection of a large number of pronuclear-stage embryos at once and a good synchrony among the different technological steps. To achieve this, large colonies of mice have to be maintained, increasing the required labor and expenses. There is often a discrepancy between the number of fertilized eggs available for microinjection and the number of synchronous foster mothers as recipients for embryo transfer (5). In order to overcome these problems, it would be useful to utilize cryopreserved pronuclear-stage mouse embryos for pronuclear microinjection. (6-9). Research in cryopreservation techniques have included studies about the type and concentration of cryoprotectant, cooling and freezing rates, seeding and plunging temperatures, warming temperatures and rates, and methods of cryoprotectant removal. Since succesful embryo cryopreservartion has been reported for several mammalian species, general reviews are available on the technological and applied aspects of the freezing of mammalian embryos (10-15). Since Rall and Fahy (12) described the first successful vitrification of mouse embryos, many investigators have studied cryopreservation of embryos by vitrification (16,17). Vitrification is defined as glass-like solidification and/or a complete avoidance of ice crystal formation during cooling. Furthermore, vitrification can eliminate mechanical damages caused by intra-or extracellular ice crystals, and chilling damages due to prolonged exposure to suboptimal temperatures. Vitrification can offer a rapid and simple alternative to cryopreserve oocytes and embryos, without using of expensive equipment. Some suitable vitrification solutions such as mixtures of dimethyl sulfoxide (DMSO), glycerol and propylene glycol (PG), ethylene glycol (EG), and trehalose and sucrose (8,9). Subsequently, glycerol, PG and EG have been reported as effective for vitrifying mouse embryos both in combination and as simple permating cryoprotectants (11,18,19). The vitrification solution contains DMSO and PG as permeable agents, polyethylene glycol (PEG) as a macro molecule compound. The inclusion of a macro molecule to the solution facilitates vitrification and also Bovine Serum albumine (BSA), Ficoll and Poly Vinly Pyrolidone (PVP) are widely used for this purpose (11,12,18). The sensitivity of embryos to cryoprotectants and cryopreservation is variable and highly dependent on the developmental stages of embryos. It has been known that the cryopreservation of PN mouse embryos has always been more difficult than that of other stages. Pronuclear-stage mouse embryos have been successfully cryopreserved either by conventional slow freezing or vitrification methods (6-9,13) reported high success rates with a new vitrification method that requires a pre-cooled metal surface (hence its name: solid surface vitrification, SSV) to vitrify matured bovine oocytes and pronuclear-stage mouse embryos (8,9,20). The method is very simple and inexpensive. Furthermore, the metal surface cooled with LN_2, the containerless cooling and the small drop size offers advantages such as a more efficient heat transfer and subsequently, increased cooling rates. In this study, *in vitro* viability of pronuclear-stage mouse embryos was examined after vitrification with three different cryoprotectants. The aim of this study was to develop an efficient and inexpensive protocol for cryopreservation and storage technique of PN embryos until their use for manipulations.

Materials and Methods

Animals, superovulation and embryo collection

Chemicals were purchased from Sigma Chemical Co (St Louis, MO, USA) unless otherwise indicated. Animal care and use procedures were in accordance with the Instutional Guide for the Care and Use of Laboratory Animals and were approved by the Research Institute of Genetic Engineering and Biotechnology (RIGEB) Animal Care and Use committee. Five-six weeks old hybrid (C57BL/6J x BALB/c) CB6F1 mice were used. The animals were housed in 14:10 hr light/dark cycle (lights on at 05.00 a.m.) at 21 ± 0.5°C and humidity of 50-60%. Females were superovulated by intra-peritoneal (i.p.) injections of 5 IU pregnant mare's serum gonadotropin (PMSG; G-4877) at 13.00 hr, followed by an i.p. injection of 5 IU human chorionic gonadotropin (hCG; Pregnyl, Organon) 48 hr later (21) then placed

individually with stud males. Successfully mated females were identified by vaginal plugs. Approximately 19-20 hr post-hCG injection oviducts were excised and PN embryos with cumulus cells were released from oviductal ampullae then transferred into drops of M2 medium supplemented with hyaluronidase (80 IU/ml) for 3 min (21). Finally, all embryos were washed three times in M2 medium, and PN embryos were selected for vitrification studies.

Vitrification and Thawing

SSV vitrification procedures were employed during the experiments. The SSV method was originally described by Dinnyes et al. (20) and slightly modified in this study. All solutions used in this study were prepared in M2 supplemented with 4 mg/ml BSA (A-9647). The effects of the vitrification solutions was tested before vitrification. Therefore PN embryos were exposed to the equilibration and vitrification solutions, and without the cooling step, moved directly into the sugar solutions, and rinsed three times M2 medium. 15 to 20 PN embryos were transferred into 50 µl drops of three different mediums: i) 4% EG (E-9129) in M2 medium at 37 ^{0}C ; ii) 4% DMSO (D-2650); iii) 4% PG (P -1009) in M2 medium at 21 ^{0}C for 12-15 min, and then the PN embryos were rinsed three times 25 ml drops of vitrification medium of i) 35 % EG, 5% Polyvinylpyrrolidone (PVP), 0.4 M Trehalose ii) 35 % DMSO, 5% PVP, 0,4 M Trehalose iii) 35 % PG, 5% PVP, 0,4 M Trehalose in M2 medium at 21 ^{0}C for 20-30 sec, respectively. PN embryos exposed to vitrification solution were either placed into a 0.3 M trehalose solution (for solution toxicity experiments), or 15-20 embryos were aspirated into a pipette with 1-2 µl vitrification solution and dropped onto a metal surface pre-cooled to -150 to -180 ºC (Figure) where they were instantaneously vitrified. After vitrifying, five drops were moved with a cooled forceps into a 1-ml cryotube (Nunc; Cat.No:343958,Roskilde, Denmark) and stored in LN_2 either for long-term (from several days to 2 to 4 weeks) or warmed after storage approximately 12-hr. All solutions in the microdrops remained transparent during cooling and warming, which is one indication that the solutions vitrified. Warming was performed by dropping the vitrified droplets from the cryotube directly into a petri-dish containing 500 ml 0.3 M trehalose solution at 37 ºC for 3 min. Warmed embryos were washed in M2 three times and kept in the last M2 drop for 10 min before transferring them into in vitro culture medium

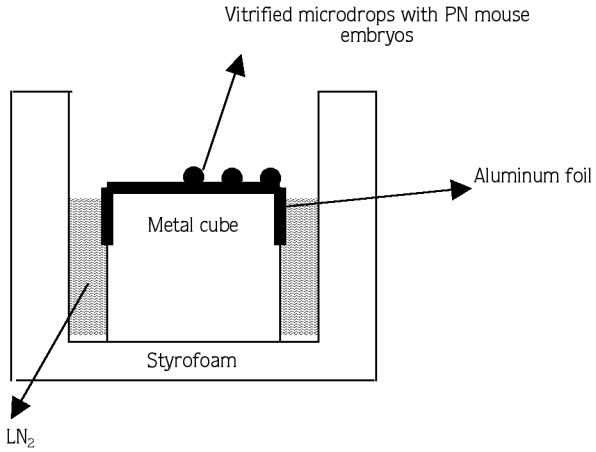

Figure. The solid surface vitrification (SSV) device: a metal cube covered with aluminum foil, partially submerged into liquid nitrogen (LN_2) (8,20).

(Potassium Simplex Optimized Medium, which is denoted KSOM) (22).

Preparation of culture media

The embryos were cultured in KSOM supplemented with BME (5 ml/ml) (M7145) and MEM (2.5 ml/ml) (B6766) amino acids (22-25). Osmolarity was measured by freeze-point depression osmometer (Osmomat 030, Gonotec GmbH) was 265 ± 10 mOsm

Embryo Culture and Evaluation

Culture plates (35-mm tissue culture plate; Nunc150288) were equilibrated with 5% CO_2 in air at 37 ºC overnight. PN embryos were transferred from M2 medium into culture drops following three times washing in culture medium and cultured in groups of 10 per 10 ml droplet of medium overlaid with embryo-tested mineral oil (M 8410) in an incubator with 5 % CO_2 in air at 37^{o}C for 5 days (120 hr post-hCG), without replacement with fresh medium. During in vitro culture, embryo development was evaluated every 24 hr 2-cell, 3-8-cell, morula, and expanded blastocyst stages were distinguished.

Statistical Analysis

Embryo developmental data was collected from at least three replicates. Vitrified- thawed PN embryos on the developmental effects in three different vitrification solutions were compared with Chi-square analysis. Differences of $P < 0.05$ or $P < 0.01$ were considered significant. The data analysis was carried out by using Graphpad Software Program (Version 2.02, Dr. Granger, LSU Medical Center).

Results

In the present study, three experimental groups and a control group were compared, but also the toxicity of the cryoprotectants were compared. No significant differences have been found between the immediate survival or cleavage rates of embryos exposed to the solutions used during the vitrification procedures and that of the nontreated controls. Experimental groups were distinguished each other by the vitrification solution used in SSV. Equilibration were performed 4% EG at 37 ºC temperature in the first group (SSV-EG), 4% DMSO at room temperature (RT) in the second group (SSV-DMSO) and 4% PG at RT in the third group (SSV-PG). After vitrification and thawing, survived embryos were cultured to blastocyst stage in KSOM. According to chi-square test results, a significant difference between the groups of SSV-EG and SSV-PG at developing rate to 2-cell stage ($P < 0.05$, Table) were determined. Similarly, SSV-PG demonstrated significant differences with SSV-DMSO and the control group at developing rate to 3-8-cell stage ($P < 0.05$). When compared the rates of developing to morula stage among the groups, significant differences between SSV-PG and the control group at $P < 0.05$ were determined; and between SSV-DMSO and SSV-PG at $P < 0.01$. Finally, the developing rates into blastocyst stage were compared and it was found that SSV-EG demonstrated significant differences with SSV-PG and SSV-DMSO at $P < 0.01$.

Discussion

The results of this study demonstrate that vitrification (SSV-EG group) can result in high developmental rates for PN mouse embryos not lower than that of the controls. Furthermore, three permeating cryoprotectants (EG, DMSO and PG) with non-permeating sugars (trehalose) were all found to be efficient for vitrification of pronuclear-stage embryos. Vitrification of pronuclear-stage embryos would be very important for transgenic studies in mice to overcome the potential discrepancies between the need and the availability of appropriate developmental stage embryos for microinjection and subsequent transfer into foster mothers. Vitrification depends on the concentration and type of cryoprotectants and equilibration time is an important element of the vitrification procedure. The cryoprotectant penetrating into the embryo is essential to achieve a vitrifiable concentration during the rapid cooling step. Following the equilibration step, a short exposure to high concentrations of cryoprotectants in the vitrification solution has a double effect on the embryos. The principal factor affecting successful cryopreservation in which only

Table. Development of vitrified PN-stage mouse embryos to the blastocyts-stage in three different solution.

Methods	Equilibration solution and time	Number of frozen PN embryos	Number of cultured PN embryos	2-cell (%)	3-8-cell (%)	Morula (%)	Blastocyst (%)
SSV-EG	4 % EG 15 min 37 ^{0}C	105*	98 (93.3)	91 (92.8)^{a}	76(77.5)^{ab}	68 (69.3)^{abcd}	52 (53.06)^{c}
SSV-DMSO	4 % DMSO 15 min RT	142*	138 (97.1)	122 (88.4)^{ab}	117 (84.7)^{a}	109 (78.9)^{abc}	43 (31.1)^{d}
SSV-PG	4 % PG 15 min RT	119*	117 (98.3)	97 (82.9)^{b}	87 (74.3)^{b}	73 (62.3)^{ad}	35 (29.9)^{d}
Control	-	-	51*	48 (94.0)^{ab}	46 (90.0)^{a}	40 (78.4)^{bcd}	28 (54.9)^{c}

EG: Ethylene glycol, PG: Propylene glycol, DMSO: Dimethyl sulfoxide, RT: Room temperature,
* Pooled data from three replicates.
$^{a\text{-}b\text{-}c\text{-}d}$Different superscripts in the same column denote significant difference at (a,b: $P < 0.05$; c,d: $P < 0.01$)

one type cryoprotectant is used of mammalian embryos one the type of cryoprotectans used. In this study, PN mouse embryos were vitrified in high concentration of EG, DMSO and PG solution by exposure. It has been reported that the cell is highly permeable to EG and DMSO may easily permeate the embryo to cause vitrification over a short time exposure (30 seconds) (26,27). The cryoproptectant also may easily efflux form the embriyonic cells without causing serious osmotic injury during warming and dilution in 0.5 M sucrose + PBS. Trehalose has been successfully used for cooling embryos in combination with other reagents, EG (8,20) and DMSO (9), PG (9). On the other hand, this study demonstrated that trehalose was also effective when utulized in combination with EG, DMSO and PG. EG has been used successfully for freezing several mammalian embryos (8,20) compared with other cryoprotectant DMSO, PG and seems to have a very low toxicity even when used in high concentration (18). Moreover, EG penetrates easily inside embryo blastomeres and restricts ice crystal formation. Recently, Yokohama et al (28) showed that EG permeates more slowly through the cellular membrane of mouse oocytes than PG, acetamide or DMSO. Szell et al (27) showed that sheep and bovine embryos are more permeable to EG than glycerol or PG. It has also been shown that EG provides better cryoprotection for mouse embryos that slowly colled to below - 60 ^{0}C than glycerol or DMSO (29). PG appears to be efficient in cryopreservation. PG is higly stable in the amorphous state, and thus, limits ice formation, it has also appears to be less toxic than DMSO and has been used in cryopreserving of early stage embryos. Mouse oocytes and PN embryos are more sensitive to cryopreservation than 2- to 8-cell or later stage embryos (30), probably due to stage-dependent changes in the membrane characteristics and other cytoplasmic factors. Cryopreservation of pronuclear-stage embryos would be very important for transgenic studies in mice to overcome the potential discrepancies between the need and the availability of appropriate developmental stage embryos for microinjection and subsequent transfer into foster mothers. Therefore, several scientists have investigated suitable techniques for cryopreservation of PN mouse embryos (6-10). The composition of the cryoprotectants is an important factor for vitrification. The extracellular, non-permeating cryoprotectants used during the SSV procedures have various beneficial effects on the vitrification process. The macromolecules of PVP elevated the viscosity of the solution without any major osmotic effects (31), and have been shown to decrease the rate of zonal disruption during vitrification (32). Trehalose is a low molecular weight non-permeating disaccharide which has an important role in preventing changes to cellular membrane during dehydration, although the exact mechanism of its action has not been understood yet (33). The use of a mixture of a fast penetrating cryoprotectant (EG), a viscosity-increasing compound (PVP) and a membrane-stabilizing sugar (trehalose) assured that even a short exposure to the solution would result in vitrification. If one vitrification method can be succesfully applied to every developmental stage of embryos, the cryopreservation of embryos will become simplier and more practical.The presence of intracellular cryoprotectants, permeating into the embryo (for example EG, DMSO, glycerol) is very important during the vitrification process (31). Ethylene glycol is a low molecular weight permeating cryoprotectant, and in addition it has an important role in stabilizing the cellular membranes during cryopreservation. In mouse oocytes it was demonstrated that EG permeates more slowly through the cellular membrane than DMSO, acetamide or PG (28).

In conclusion, in this study it was demonstrated that pronuclear-stage mouse embryos were succesfully vitrified in a EG, DMSO and PG under the same conditions and dilutions via SSV techniques. This study has shown that, EG, DMSO and PG with trehalose can be used effectively as a cryoprotective agent in the quick freezing of pronuclear-stage mouse embryos. Additionally, it has been demonstrated that there is no statistical difference between EG, DMSO and control groups at cleavage rates. However, EG group had the highest blastocyst rate when comparing with the other groups including the control. Furthermore, all vitrification procedures are carried out at room temperature. Finally, additional studies are needed to optimize the SSV method in further stage mouse embryos.

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