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The Effect of Solid Surface Vitrification (SSV) Versus Classic Vitrification Technique on Survive Rate of *in vitro* Produced Bovine Blastocysts

[1]Tolga Akkoc, [1]Ali Cihan Taskin, [1]Arzu Tas Caputcu, [1]Sezen Arat and [1, 2]Haydar Bagis
[1]TUBITAK, MRC, Genetic Engineering and Biotechnology Institute (GEBI), Gebze, Kocaeli, Turkey
[2]Department of Medical Genetic, Faculty of Medical, Adiyaman University, Adiyaman, Turkey

Absract: The aim of this study was to compare Solid Surface Vitrification (SSV) technique and classic vitrification technique in *in vitro* produced 8 days old bovine blastocysts. Cryopreservation of mammalian embryo has great importance for genetic resources conservation, embryo transfer, veterinary and clinical reproductive biotechnology and animal assisted reproductive technologies. Immature oocytes were maturated then fertilized with frozen-thawed bull semen and cultured until blastocyst stage in commercial sequential culture medium for 8 days. Blastocysts were vitrified in two different groups as SSV and classic vitrification and non-vitrified blastocysts were used as control group. After vitrification, vitrified blastocysts were warmed and cultured for 1 day. For this aim, blastocyst viability rate and median cell number were investigated. The blastocyst viability rate that vitrified by classic vitrification (34.8%) were found to be lower than those vitrified by SSV (82.6%) and control group blastocysts (100%). However, median cell numbers of vitrified-warmed blastocysts were found higher in SSV (124) than classic vitrification (104). Median cell number of control group was detected as 213. As a result, blastocyst viability rate and median cell number in SSV group was higher than classic vitrification group, there was a significant difference between SSV and classic vitrification group ($p<0.05$).

Key words: SSV, vitrification, immature oocyte, bovine blastocyst, embryo transfer, Turkey

INTRODUCTION

Cryopreservation of pre-implantation embryos plays a key role in further development of embryo freezing technology. Embryo vitrification is a simple method that whole solution the biological sample vitrifies completely (Rios *et al*., 2010). Since, the first successful cryopreservation studies of mouse 8 cell embryos (Rall and Fahy, 1985), several methods have been proposed for cryopreservation of other mammalian species embryos including mouse (Ishimori *et al*., 1992; Kassai *et al*., 1990; Rall, 1987; Scheffen *et al*., 1986; Valdez *et al*., 1992), rabbit (Kasai *et al*., 1992; Kobayashi *et al*., 1990; Smorag *et al*., 1989), sheep (Schiewe *et al*., 1991), porcine (Dobrinsky and Johnson, 1993) and bovine (Douchi *et al*., 1993; Kuwayama *et al*., 1992; Massip *et al*., 1987; Yang *et al*., 1992; Van Der Zwalman *et al*., 1989). Vitrification that causes solidification of a solution without ice crystal formation is an alternative method to freezing without using expensive equipment (Bagis *et al*., 2005, 2009; Li *et al*., 2002; Somfai *et al*., 2009). This offers a rapid and simple alternative technique to cryopreserve oocyte and embryos. In vitrification technique both intracellular and extracellular ice crystal formation are prevented by using high concentrations of cryoprotectans and high cooling and warming rates (Vajta, 2000; Wolfe and Bryant, 1999) and extreme elevates viscosity druing cooling (Fahy *et al*., 1984; Kaidi *et al*., 2001). However, cryoprotectans causes toxic effect and osmotic pressure when used at high concentrations that occurs negative effect on survivability of embryos (Bagis *et al*., 2004, 2005; Yavin and Arav, 2007). It has been suggested that increasing cooling rate by decreasing the sample volume allows a reduction in cryoprotectant concentration.

Reviews and literatures including freezing studies in farm and domestic animals have already been published (Massip, 2001). Recently, most research has been interest to cryopreservation of IVP bovine embryos and also these embryos are more sensitive to cryopreservation and have significantly reduced pregnancy rates after embryo transfer than *in vivo* produced bovine embryos (Diez *et al*., 2001; Pugh *et al*., 2000; Sommerfeld and Niemann, 1999).

Cryopreservation of bovine blastocysts have been successfully done either by SSV or classic vitrification

Corresponding Author: Sezen Arat, TUBITAK, MRC, Genetic Engineering and Biotechnology Institute (GEBI), Gebze, Kocaeli, Turkey

techniques (Saito *et al*., 1994). SSV is a simple method for using a pre-cooled (-150 to -180°C) metal surface with LN2 that was performed by vitrifying matured oocytes, Pronuclear stage (PN) mouse embryos, pig embryos and goat oocytes with high rates of survival (Bagis *et al*., 2002, 2005, 2009; Begin *et al*., 2003; Dinnyes *et al*., 2000). SSV also allowed Rhesus monkey oocytes for the 1st time (Dinnyes *et al*., 2004). In this method, using small drops size solution for vitrifying oocyte or embryos offers advantages for both effective heat transfer and more less toxic effect of cryopretectans. This method has been tested further by other group for PN stage mouse embryos resulting in the birth of progeny (Bagis *et al*., 2002).

Classic vitrification is an alternative cryopreservation technique that performed *In Vitro* Maturated (IVM) and *In Vitro* Fertilized (IVF) bovine 6-8 days old bovine blastocysts (Saito *et al*., 1994). In this method, embryos were exposed to vitrification solutions included increasing molarities of cryoprotectants then immediately after exposure to the final solution, 1-10 embryos were loaded into 0.25 mL. French type straws (Code Art-005592) and finally loaded straw plungs into LN_2 (Saito *et al*., 1994). In comparison to SSV, the classic vitrifiacion seems to be need more amount of vitrification solution that may cause more risk for toxicity of cryoprotectans however, heat transfer during vitrification is more less.

The objective of this study was to compare directly the effects of SSV and classic vitrification procedures of *In Vitro* Produced (IVP) 8 days old bovine blastocyst and to investigate the total cell number of re-expanded blastocysts subsequent *in vitro* culture post thawing.

MATERIALS AND METHODS

All chemicals used in this study were purchased from Sigma Chemical Co. (St. Luis, MO, USA), unless otherwise indicated.

Bovine oocyte recovery: Bovine ovaries were collected from a local abattoir within 10 min post-slaughter and placed into thermos bottle containing physiological saline solution included 0.9% Sodium Chloride (NaCl; S-5886) at 26-33°C, immediately after shipped to the laboratory within 3-4 h after collection. Ovaries were washed twice with physiological salin solution (0.9% NaCl) at 37°C. Cumulus Oocytes Complexes (COCs) were collected from 2-8 mm in diameter of ovarian follicles using 18 gauge needle by aspiration method and pooled into 50 mL^{-1} plastic flask (TPP-91050) containing 5 mL^{-1} TL-HEPES solution supplemented with 0.3% BSA (A-6147). Only oocytes with homogenous cytoplasm surrounded by compact, dense 2-4 layer cumulus cell layers were selected under stereomicroscope for IVM.

***In vitro* maturation:** Selected COCS were washed three times in TL-HEPES medium and placed into IVM medium. The medium used for oocyte IVM was TCM-199 (Earle's salts and with L-glutamine; SIGMA (M-5017) supplemented with 2.2 g L^{-1} sodium bicarbonate (S-5761), 10% FCS (GIBCO-10500), 5.5 μL mL^{-1} sodium pyruvate (P-4562), 1% v:v penicillin-streptomycin (10.000 U mL^{-1} penicillin G (P-4687), 10, 000 μL mL^{-1} streptomycin (S-1277), 5.0 μg mL^{-1} bovine Luteinizing Hormone (bLH) (L-5269), 0.5 μg mL^{-1} bovine Follicle Stimulating Hormone (bFSH) (F-2293) and 10 ng mL^{-1} Epidermal Growth Factor (EGF) (E-4127) 10 ng μL^{-1} and Insulin Growth Factor (IGF) (I-3769) 100 ng μL^{-1} (Bagis *et al*., 2009; Cevik *et al*., 2009). Selected COCs were placed into 500 μL^{-1} maturation medium in 4 whell dishes (176740-Nunc Roskilde, Denmark) under 300 μL^{-1} mineral oil (M-8410). Maturation of COCs were performed by incubation for 24 h at 38. 5°C in air containing CO_2 5% with saturated high humidity. After maturation COCs having expanded cumuls cells and homogenous ooplasm were selected for IVF.

Sperm preparation and *in vitro* fertilization: After maturation in culture, oocytes with expanded cumulus cells were used for fertilization. COCs were washed twice in a fertilization medium (Quill's Advantage Fertilization Media®-1020) supplemented with 6 mg mL^{-1} BSA-FAF then transferred into 44 μ L^{-1} fertilization drops (10-12 oocytes/drop). A stock of Percoll (P-4937) solution was prepared at a 9:1 mixture of percoll and a x10 stock of salt solution. (2, 3375 g NaCl, 0, 115 g Potassium Chloride (KCl; P-5405), 0, 0175 g potassium dehdyrogen phosphate (KH2PO4; Applichem A-2945) and 1, 19 g Hepes (H-6147) in 50 m L^{-1} water for embryo transfer (W-1503). The 90% percoll gradient solution was prepared by diluting of x10 stock solution. To prepare the 45% Percoll solution, 2 m L^{-1} of 90% percoll solution was with 2 m L^{-1} of Sperm washing medium (1:1 ratio) (Sage sperm washing medium-1006). Frozen thawed bull semen was used in this study. Two straw of frozen bull spermatozoa were thawed at 37°C in distile water bath for 1 min. The thawed bull spermatozoa were layered onto discontinuous percoll density gradient (2 m L^{-1} of 45% over 2 m L^{-1} of 90% v:v in sperm washing medium) in a 15 mL^{-1} conic tube (TPP-91015) and centrifuged at 700×g for 15 min at room temperature. After centrifugation, supernatant were removed than approximately 10 μL^{-1} pellet of spermatozoa were layered onto lam for visually evaluate using a phase

contrast microscope at a magnification of 400x. The concentration of spermatozoa was adjusted to 2×10^6 spermatozoa mL^{-1} into IVF medium.

***In vitro* culture:** After 18 h of sperm and oocyte incubation, all embryos were washed twice with TL-HEPES solution and vortexed for 3 min in 100 μL^{-1} of TL-HEPES solution for removal of cumulus cells. Then cumulus free embryos were washed twice and 12-15 embryos were cultured in 30 μL^{-1} droplets of Quins Advantage Cleavage Medium (QACM) (8 mg mL^{-1} BSA-FAF) for 72 h and in Quins Advantage Blastocyst Sequental Medium (QABM) (4 mg mL^{-1} BSA-FAF+ 5% FCS) for additional 4 days. The cultures of the embryos were performed in incubator at 38.5°C in 5% CO_2 and humidified air. At 8 days old blastocyst stage embryos were used in vitrification and for control group.

Group 1: The 1st vitrification system was SSV (Bagis *et al*., 2009). After *in vitro* culture, briefly 4-5 blastocyst stage embryo were rinsed three times in Base Medium (BM) TCM-199 with Earles salt (M-2520) supplemented with 20% (v:v) FBS then suspended into a 50 μL^{-1} of equilibration medium that contains 4% v:v Ethylene Glycol (EG; E-9129) in BM at room temperature for 12 min. After embryos were transferred into a 20 μL^{-1} of vitrification medium containing of 35% EG, 5% Polyvinylpyrrolidone (PVP40; P-0930) and 0.4 M trehalose (T-0167) in BM at room temperature and rinsed two times for 30 sec. A group of 1 embryo was loaded into transfer pipette containing 1-2 μL^{-1} of vitrification solutions and dropped on a steel surface covered aluminum foil pre-cooled to around -150 to -180°C. Vitrified embryos were waited for 30 min onto pre-cooled metal surface. Thawing of vitrified blastocyst were carried out by transferring vitrified drops into 500 μL^{-1} warming solution including 0.3 M trehalose in BM at 38°C for 3 min. After 3 min, warmed blastocysts were subsequently transferred for 8 min periods into 500 μL^{-1} BM at 38°C and rinsed three times by exposing to the 500 μL^{-1} same solution. The survive vitrified thawed blastocysts were subjected to *in vitro* culture in QABM supplemented with 4 mg mL^{-1} BSA-FAF+5% FCS in incubator at 38.5°C in 5% CO_2 and humidified air for 1 day.

Group 2: The 2nd vitrification system was classic vitrification method (Saito *et al*., 1994) After *in vitro* culture, 4-5 blastocysts were rinsed three times in Base Medium (BM) M-PBS in 38°C then exposed to 1st Vitrifiction Solution (VS1) supplemented with 10% glycerol (G-5516), 0.1 M sucrose (S-1888), 0.1 M xylose (X-3877) and 1% polyethylene glycol (P-2139) in M-PBS for 5 min at room temperature. Subsequently, embryos were transferred into 2nd Vitrification Solution (VS2) supplemented with 10% glycerol, 10% EG, 0.2 M sucrose, 0.2 M xylose and 2% polyethylene glycol in M-PBS for 5 min at room temperature. Finally, embryos were transferred into third Vitrification Sollution (VS3) supplemented with 20% glycerol, 20% EG, 0.3 M sucrose, 0.3 M xylose and 3% polyetylene glycol in PBS at room temperature and loaded into 0.25 m L^{-1} French type straw a column (5 cm) of 0.5 M sucrose, 1 cm Air bubble, 3 cm VS3 solution included embryos, 1 cm air bubble, 3 cm of 0.5 M sucrose. Subsequently, loading embryos into straw both sides of straw heat-sealed then it slowly plunged into the LN2. Exposing blastocysts into VS3 solution, loading straws and plunging straws into LN2, respectively were performed maximum in 1 min.

Vitrified blastocysts were warmed after 30 min warming was performed by plunging the straw into 20°C water bath until the crystallized warming solution melt. Melting time was cost approximately 10 sec. The contents of straw was emptied in a free petri dish and vitrified warmed blastocysts were collected then transferred into a dilution solution containing 0.5 M sucrose supplemented with 20% FCS in M-PBS for 5 min. Subsequently, embryos were transferred into 0.25 M sucrose supplemented with 20% FCS in M-PBS for 5 min. All dilution process was performed in room temperature (23-25°C). When all vitrified blastocysts were warmed they rinsed three times in TCM-199 supplemented with 20% FCS then transferred into 30 μL^{-1} droplets of the same solution. Culture was performed in incubator at 38.5°C in 5% CO_2 and humidified air for 1 day.

Fluorescent staining of embryonic nuclei: Throughout *in vitro* culture of vitrified and warmed blastocysts, the progression of expandation stage was observed under inverted microscope (Zeiss axiovert 35 M). Total nuclei numbers of expanded blastocysts were evaluated using bisbenzimide (Hoechst 33342; B-2261) fluorescent DNA staining technique (Bagis *et al*., 2003; Rall, 1987). Each blastocyst was exposed 10 μL^{-1}, drop 5 μmL^{-1} Hoechst 33342 for 10 min at room temperature in the dark.

Experimental design: In order to test the effects of vitrification methods for survivability and expandation rate the day 8 of blastocyst stage IVP bovine embryos, three major experiments were carried out. In each experiment blastocysts were randomly distributed into three groups; control group, SSV group and classic vitrification group.

Statical analysis: The experiments were performed at least three replication. The data were analyzed using SPSS (Statistical Package Social Sciences, Version 10.0) for Windows (MS). Blastocysts viability rate and total blastocysts nuclei number was analyzed by t-test. The p-value used to determine significance in all test was 0.05.

RESULTS AND DISCUSSION

Vitrification of bovine blastocysts: Due to previous experimental data of *in vitro* fertilization and *in vitro* culture studies, blastocyst stage embryos were selected and vitrified in two different vitrification procedures. Subsequently, vitrified blastocysts were thawed. All thawed blastocysts were cultured 18 h than their both expandation and hatching rate were compared. Survival of vitrified-thawed blastocysts was determined as by checking morphologically quality indicates homogeneous ooplasm, specific perivitellin area and proper cytoskeleton structure. The developmental competence was compared and showed in Table 1. The viability rate of SSV group vitrified thawed and cultured blastocysts 19/23 (82.6 %) were higher than classsic vitrification group blastocysts 8/23 (34.8 %). However, the viability rates of un-vitrifed blastocysts as control group were higher than both two experimental groups 17/17 (100 %). Furthermore, hatching rate of vitrified with SSV method and thawed and 4/23 (17.4 %) of cultured blastocysts were found to be higher than classic vitrification group 1/23 (4.3%) in Table 1. However, hatching rate of un-vitrified blastocysts as control group was higher than both two experimental groups 11/17 (64.7%). There was a significant difference between SSV technique, classic vitrification technique and control group.

Florescent staining of embryonic nuclei: Nuclear staining for assessment of vitrified-thawed and in consequence of *in vitro* culture expanded blastocysts were performed with Hoechst 33342 in 5.5 μ mL^{-1} concentrations for 10 min at room temperature in the dark. In SSV median, cell number were found higher than classic vitrification technique. Median cell numbers were 124 and 104 in both experimental groups, respectively. Median cell number of control group was detected as 213. There was a significant difference between SSV technique and control group. The present study demonstrated that a novel and simple vitrification method called SSV can be used to successfully vitrify 8 day old bovine blastocysts, resulting high rates of survival, *in vitro* development, re-expendation rates and resulting high total nuclei number.

In the present study, researchers demonstrated that SSV technique resulted in higher survival and expandation rates in comparison to classic vitrification technique. Nevertheless, SSV technique showed higher ratio of total cell number than classic vitrification technique.

In recent studies, clearly showed that vitrification of mammalian gametes, embryos and tissues is a promising technique for cryopreservation studies. With vitrification techniques in order to inhibit the ice-crystal formation, high concentrations of cryoprotectant agents such as (DMSO, EG, PEG, trehalose, sucrose, xylose and glycerol) can be used. This technique leads to development of a solid, glass like so called vitrified state which water is solidified on the opposite not expanded. In addition for decrease the toxic effect of cryoprotectant agents on vitrified embryo, they should be exposed to cryoprotectant solution for a minimum period of time and a minimum volume at vitrification solution.

In the present study, the researchers compared SSV and classic vitrification technique as a two different vitrification techniques. The volume sample of cryoprotectant solutions is a factor that affects the viability after vitrification. First one is decreasing the volume of cryoprotectan solution sample increases the cooling rate and also reduces intracellular ice crystal formation (Arav *et al*., 2002; Rios *et al*., 2010) and other one is heat transfer ratio.

Both SSV and classic vitrification techniques are inexpensive and easy to perform and manipulate than other vitrification and slow freezing methods. Previous studies from the laboratory demonstrated that SSV technique is a suitable in order to produce transgenic mouse from vitrified-warmed PN mouse embryos without major reduction (Bagis *et al*., 2002). In other studies,

Table 1: Number of blastocysts expanded and hatched *in vitro* from fresh, SSV, classic vitrified-thawed 8 days old bovine blastocyst stage embryos

Treatments	No. of blastocysts vitrified-thawed	Viability rate of thawed blastocysts (%)/no. thawed blastocysts cultured*	No. expanded of thawed blastocysts (%)/no. thawed blastocytst cultured	No. hatched of thawed blastocytsts (%)/no. thawed blastocysts cultured
SSV	23	19/23 (82.6)[a]	12/23 (52.2)[a]	4/23 (17.4)[a]
Classic vitrification	23	8/23 (34.8)[b]	2/23 (8.7)[b]	1/23 (4.3)[b]
Non-vitrified (control)	17	17/17 (100.0)[a]	6/17 (35.3)[a]	11/17 (64.7)[a]

*No. of continued vitality of normal, expanded and hatched blastocysts

inclusive comparison different cryopreservation techniques on mouse PN embryos (Bagis *et al*., 2009). As a result of all studies, SSV technique has given better survival and development rates than other cryopreservation techniques.

The 1st successful vitrification of bovine embryos were carried out by Massip. SSV vitrification technique were applied to vitrify matured bovine oocytes, mouse PN embryos, pig embryos, goat oocytes and biopsied 8 blastomere stage mouse embryos with high survival rates (Bagis *et al*., 2002, 2009; Barayani *et al*., 2005; Begin *et al*., 2003; Li *et al*., 2002; Somfai *et al*., 2009).

Vitrification of *in vitro* produced bovine blastocysts causes vulnerable to cryo-damage due to intracellular and membrane detects caused by exchange of water and cryoprotectant agents between the intracellular and extracellular environment (Bagis *et al*., 2009; Dinnyes *et al*., 1999). Chilling injuries due to intracellular and extracellular ice crystal formation, solution effects and also osmotic shock are the main adverse consequences following cryo-procedures (Pereira and Marques, 2008). The success of the vitrification, mainly depends on the type and concentration of cryoprotectant (exp: EG, xylose, PEG and trehalose), freezing and thawing rate, techniques and also device (Bagis *et al*., 2005; Rall, 1987).

The researchers compared two vitrification techniques as SSV and classic vitrification technique used to vitrify 8 days old bovine blastocysts and found various differences between them. The blastocyst viability rate of vitrified by classic vitrification technique method (34.8%) were found to be lower than those vitrified by SSV method (82.6%) and control group blastocysts (100%) ($p<0.05$). Nevertheless, median cell numbers of vitrified thawed blastocysts were found higher in SSV technique (124) than classic vitrification technique (104). Median cell number of control group was detected as (213). There was a significant difference between SSV and control group. ($p<0.05$).

The volume sample of cryoprotectant solutions is a factor that affects the viability after vitrification. First one is decreasing the volume of cryoprotectan solution sample increases the cooling rate and also reduces intracellular ice crystal formation (Arav *et al*., 2002; Rios *et al*., 2010) and other one is heat transfer ratio.

CONCLUSION

The study shows that blastocyst viability rate and total cell number in SSV technique was higher than classic vitrification technique.

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