Effect of the Artificial Shrinkage on the Development of the Vitrified Bovine Embryos

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ABSTRACT

This study was conducted to find out the effects of artificial shrinkage (AS) on post-thaw development of bovine embryos. The blastocoelic cavity of blastocyst was punctured to remove its fluid contents and then incubated in the holding medium (HM) for 10 min. The punctured and non-punctured (control) blastocysts were equilibrated in vitrification solution 1 (VS1; TCM-199+20% FBS+10% EG) for 5 min and vitrification solution 2 (VS2; TCM199+20% FBS+35% EG+5% PVP+0.5 M Sucrose) for 1 min and vitrified by direct dropping into the liquid nitrogen. Vitrified blastocysts (punctured and control) were thawed and cultured *in vitro* (12 hr) for studying survival and hatching rates. The levels of shrinkage were measured by the volume of the blastocyst during equilibration in VS1 (at 1, 3 and 5 min of equilibration) and VS2 (at 30 and 60 sec of equilibration) that was considering the volume of non-punctured blastocyst in HM as 100%. The levels of shrinkage were higher in punctured group (62.4, 64.6, 64.3% at 1, 3 and 5 min in VS1; 50.6 and 52.7% at 30 and 60 sec in VS2) than control group (84.8, 86.6, 86.4% at 1, 3 and 5 min in VS1; 72.1 and 68.8% at 30 and 60 sec in VS2), but within each group the levels of shrinkage were similar. The survival (90.9%) and hatching (50.0%) rates of vitrified blastocysts at 12 hr post-thaw were higher in punctured group than that in control group (76.9% and 0.0% respectively). We confirmed that vitrification solutions (VS1 and VS2) have no toxic effect on the survival of blastocysts because the survival rates of blastocysts exposed to VS1 and VS2 for 24 hr were similar between punctured and control groups (94.3 vs. 96.0%; $p>0.05$). In conclusion, the preliminary data show that AS of blastocyst may improve survival and hatching rate after thawing.

(Key words : artificial shrinkage, vitrification, bovine, blastocyst, microdroplet)

INTRODUCTION

Cryopreservation of embryos has proven important for the best use of supernumerary embryos in assisted reproductive technology of farm animals. Though cryopreserved bull spermatozoa have been used since the 1950s for the artificial insemination of cattle (Foote *et al.*, 1993), the use of cryopreserved embryos is relatively new. Many reports have been published to optimize cryopreservation protocol of mammalian embryos focusing on pretreatment, exposure time and temperature of the blastocyst to the vitrification solution in order to minimize the chemical toxicity of the solution (Papaioannou *et al.*, 1988; Zhu *et al.*, 1993), minimization of intracellular ice formation, prevention of fracture damage (Kasai *et al.*, 1996) and osmotic swelling during the removal of the cryoprotectant (Kuwayama *et al.*, 1992). However, the viability of post-thaw vitrified blastocysts is still low particularly when expanded blastocysts are vitrified than early stage blastocyst. Several reports have been suggesting that loss of viability after vitrification of blastocysts could be attributed to physical damages resulting from ice formation during the cooling and thawing procedures. It is probable that inadequate permeation of the cryoprotectants or a too slow cooling rate leads to intra-blastocoelic ice formation during freezing (Andrabi, 2007). Many studies have been suggested that mechanical damages caused by ice crystal formation could be avoided by reducing the fluid content of the blastocoele of more developed stage blastocyst before vitrification by puncturing the blastocoele cavity with needle (Vanderzwalmen *et al.*, 2002), micropipetting without puncturing the zona and trophectoderm (Hiraoka *et al.*, 2004). The removal of fluid from blastocoele cavity prior to

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vitrification can be improved post-thaw survival, re-expansion, cell proliferation, DNA damage and pregnancy rates (Son *et al.*, 2003; Hiraoka *et al.*, 2004). Therefore, this experiment was aimed to check the effect of artificial shrinkage on post-thaw development of Hanwoo blastocysts derived from *in vitro* fertilization.

MATERIALS AND METHODS

1. *In Vitro* Embryo Production

In vitro embryo production was performed as described (Jeong *et al.*, 2009). Briefly, oocytes were aspirated from 2~8 mm antral follicles by 18 G needle fitted with a vacuum pump. Oocytes having at least ~3 layers of intact cumulus cells and homogenously granulated cytoplasm were used for *in vitro* maturation (IVM). Selected compacted oocyte cumulus (COCs) were washed 2~3 times in TL-HEPES (Tyrode's lactate hepes) before final washing in the maturation medium (TCM-199 supplemented with 10% FBS, 1 μg/ml β-estradiol, 10 μg/ml FSH, 0.6 mM cystein and 0.2 mM Na-pyruvate) and were transferred into IVM medium for 22~24 hr. *in vitro* matured COCs were fertilized with frozen-thawed spermatozoa from the same batch of semen of Hanwoo bulls. Thawed sperm were washed in Dulbecco's phosphate buffered saline (GIBCO, USA) and capacitated by incubating with 20 μg/ml heparin sodium salt in IVF medium (Tyrode's lactate solution supplemented with 6 μg/ml Bovine serum albumin, 22 μg/ml sodium pyruvate and 100 IU/ ml penicillin G/streptomycin) for 15 min. The capacitated motile spermatozoa were finally diluted in IVF medium at 1×10^6 spermatozoa/ml. The matured COCs were cocultured with capacitated sperm for 18~20 hr. Cumulus cells of the presumptive zygotes were removed by pipetting in TL-Hepes in a 35×10 mm dish (SPL, seoul Korea). The presumptive zygotes were placed in a well of 4-well dish (SPL, Seoul, Korea) containing 700 μL modified CR1-aa medium (Martino *et al.*, 1996) supplemented with 44 μg/ml Na-pyruvate, 14.6 μg/ml glutamine, 10 μL/ml penicillin/streptomycin, 3 mg/ml BSA and 310 μg/ml glutathion and incubated for 3 days (IVC-I). After 3 days of IVC-I, the embryos were cultured in the same medium addition BSA was replaced with 10% FBS for additional 4 days (IVC-Ⅱ). Embryos were cultured in an atmosphere of 5% CO_2 in air at 38.5℃ and maximum humidity during the whole process of IVP.

2. AS of Blastocysts by Micromanipulation Technique

Blastocysts were AS before vitrification mechanically as described earlier (Desai *et al.*, 2008). Briefly, blastocysts were carefully positioned on the holding pipette so that the inner cell mass was located at either the 12 or 6 o'clock position and the thinnest area of trophectoderm was at the 3 o'clock position. An ICSI needle was slowly inserted into the blastocoele cavity through the cellular junction of the trophectoderm cells (Fig. 1) and fluid was allowed to exodus maintaining very slight negative pressure until it shrank. This process was usually completed within 5 min of the AS procedure.

3. Vitrification and Thawing of Blastocysts

Blastocysts were vitrified by microdroplet vitrification method as described previously with minor modifications (Dinnyes *et al.*, 2000; Nedambale *et al.*, 2006). The punctured or control blastocyst were equilibrated in the equilibration solution VS1 (TCM199+20% FBS+10% EG) for 5 min before being rinsed in vitrification solution VS2 (TCM-199+20% FBS+35% EG+5% PVP+0.5 M Sucrose) for 30 sec. They were then either placed into a solution (composition solution for toxicity control) or directly plunged into the liquid nitrogen (LN_2) using micropipette. The vitrified droplets were moved with a LN_2 cooled forceps into a 2 ml cryovials either for long-term storage or immediately thawed by being dropped into a 38.5℃ in TCM-199 containing 0.3 M sucrose and 20% FBS for 1 min, followed by serial dilutions in 0.15 M sucrose solution for 5 min to remove cryoprotectants. Then, embryos were cultured into IVC-2 medium for up to 12 hr.

4. Comparison of Volume Change of Blastocysts

The volume of blastocysts (punctured or control) were measured after 10 min incubation in holding medium (TCM-199+20% FBS) at 3 time point during equilibration VS1 (at 1, 3 and 5 min) and 2 time points during vitrification VS2 (at 30 and 60 sec). Embryo diameters, excepting ZP, were measured from the same images on the screen of the monitor using scale bar micrometer, which was previously calibrated on a ×40 ob-

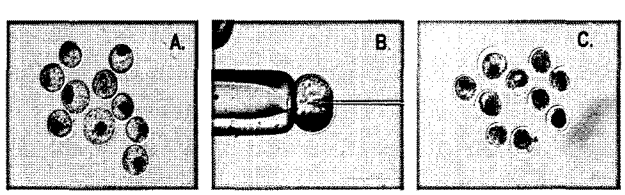

Fig. 1. Removing of blastocoele fluid using a micromanipulator: (A) Day-7 intact blastocysts, (B) Puncture by micro-manipulator and (C) Reduced volume of punctured blastocysts.

jective and ×10 eyepieces using an epifluorescence microscope (Olympus IX71, Tokyo, Japan). Volume calculation applied to formula of (V=3/4* πr^3) measuring radius with diameter.

5. Analysis of Total and Apoptosis Cell Number

Blastocyste were removed from the culture medium, washed 2~3 times in PBS-PVP and fixed in a 4 well dish containing 700 μL 4% (w/v) paraformaldehyde in PBS-PVP for 1 hr at room temperature. After fixation, embryos were stored at 4℃ until TUNEL assay performance. The TUNEL assay was performed according to the manufacturer protocol using the In Situ Cell Death Detection Kit (Fluorescence; Roche Diagnostics Corp.; Indianapolis, IN, USA; Cat. 1684795). Briefly, the fixed embryos were washed twice with PBS-PVP before being incubation in permeabilization solution [0.5% (v/v) Triton X-100, 0.1% (w/v) sodium citrate] for 30 min at room temperature. After permeabilization, the embryos were washed twice in PBS-PVP and then incubated in fluorescence-conjugated dUTP and terminal deoxynucleotide transferase (TUNEL reagent, Roche) for 1 hr at room temperature in the dark. The TUNEL stained embryos were then washed in PBS-PVP and counterstained with Hoechst 33342 (10 μg/ml) in PBS-PVP to label all nuclei for 10 min at temperature in the dark room. The blastocysts were then washed twice in PBS-PVP to remove excess Hoechst 33342 and mounted on glass slides under coverslip to evaluate the nuclear configuration. Cell numbers of each blastocyst were counted using epifluorescence microscope (Olympus IX71, Tokyo, Japan) equipped with a mercury lamp. TUNEL positive cell was indicated by a bright red fluorescence indicating apoptotic cells and the total cell number was determined by green/blue fluorescence. Embryos exposed to the TUNEL assay were randomly selected from a pooled population.

6. Statistical Analysis

Chi-squared test was used to determine differences between two vitrification procedures as well as between blastocyst and hatching blastocyst rate. Data for embryo diameters were expressed as mean±SE and assessed by analysis of variance (ANOVA) using Kruskal-Wallis test. Differences between groups at $p<0.05$ were considered significant.

RESULTS

Total 132 bovine blastocysts were vitrified and thawed in this study. The results obtained were depicted in Table 1~3 and Fig. 2~4 under the following subtopics;

1. Comparison of Volume Change according to AS with Control Group

The levels of shrinkage were measured by the volume of the blastocyst during equilibration in VS1 (at 1, 3 and 5 min of equilibration) and VS2 (at 30 and 60 sec of vitrification) considering volume of non-punctured blastocyst in HM as 100%. The levels of shrinkage were higher in punctured group compared to control group at each time point during equilibration and vitrification ($p<0.01$), but within each group the levels of shrinkage at different time points were not differed (Fig. 2).

2. Effect of Vitrification Solution on Survival Of Blastocyst

Blastocysts (punctured and control) exposed to vitrification solutions were cultured in the IVC-2 medium to test whether these solutions have any toxic effect on survival of blastocyst. Survival rates following 24 hr *in vitro* culture were similar ($p>0.05$) between punctured and control groups as shown in Table 1.

3. Effect of AS on Post-thaw Development of Vitrified Blastocysts

Survival and hatching rates at 12 hr *in vitro* culture following vitrification and thawing were higher in punctured group

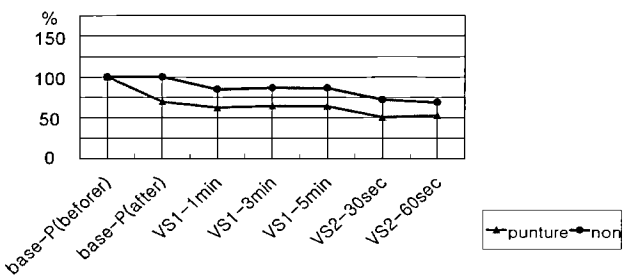

Fig. 2. Changes of embryo volumes by cryoprotectants exposure after artificial shrinkage.

Table 1. Toxic effect of vitrification solutions on survivability of post-thaw *in vitro* culture embryos

Exposed solution	Artificial shrinkage	No. of blastocyst used	No. (%) of blastocyst developed to re-expanded at 24 hr post-thawing
VS1+VS2	Control	30	24 (96.0)[a]
	Punctured	35	33 (94.3)[a]

*Embryos were exposed to VS1 for 5 min followed by VS2 for 1 min

[a]Values with same superscript in the same column were not significantly different ($p>0.05$).

Table 2. Effect of artificial shrinkage before vitrification on the viability and hatching rates of vitrified embryos

Treat-ment	No. (%) of blastocyst recovered/ vitrified	No. (%) of blastocyst at 12 hr	
		Survived/ recovered	Hatching/ recovered
Control	32/35 (91.4)	25/32 (78.1)*	0/32 (0.0)**
Punctured	29/31 (93.5)	27/29 (93.1)	15/29 (51.7)

*$p<0.05$, **$p<0.01$

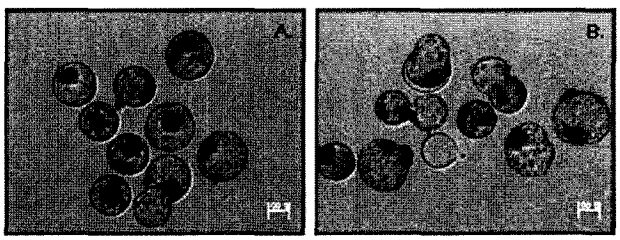

Fig. 3. Development of vitrified blastocyst after 12 hr post-thaw *in vitro* culture: Blastocyst of (A) control (non-punctured) and (B) punctured group.

compared to control group (Table 2, Fig. 3).

4. Effect of AS on Quality of Post-thaw Blastocysts

We evaluated total and apoptotic cell number in expanded post-thaw blastocyst by Hoechst and TUNEL staining (Table 3, Fig. 4). Total cell numbers per blastocyst was independent of AS ($p>0.05$). However, AS reduced apoptotic cell number in post-thaw punctured and non-frozen groups than that in control group ($p<0.01$). Also the apoptosis cell number was not different between punctured and non-frozen groups.

DISCUSSION

Our results demonstrated that AS method is very effective for promotion of survival and hatching rate, and lowering apoptotic cell number following *in vitro* culture of frozen-thawed embryos. This study provides further evidence for the potential benefit of blastocoele fluid reduction prior to vitrification.

Reduction of blastocoele fluid prior to vitrification prevents embryos from mechanical injuries caused by ice-crystal usually formed during normal cryopreservation (Cho *et al.*, 2002b; Vanderzwalmen *et al.*, 2002; Son *et al.*, 2003). During the process of cryopreservation, cells are exposed to subphysiological temperatures and are thus vulnerable to chilling injury be

Table 3. Cell number (Mean±SE) of post-thaw *in vitro* cultured (24 hr) blastocyst

Treatments	Total cells	Apoptotic cells
Non-frozen	171.8±32.1[a]	0.6±0.5[c]
Control	143.4±27.1[b]	8.0±6.3[c]
Punctured	168.6±31.1[b]	0.3±0.7[d]

[a~d] Different superscript within same column were significant difference ($p<0.01$).

All of groups were counted cell number in each 10 blastocysts.

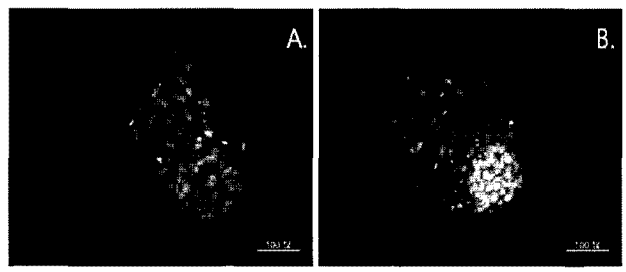

Fig. 4. Cell apoptosis phenomenon after freezing and thawing that appear after tunnel staining. (A) punctured group and (B) control group.

cause their lipids undergo phase transition (Ghetler *et al.*, 2005). The lipid composition of the membrane strongly influences its resistance to thermal stress (Arav *et al.*, 2000; Zeron *et al.*, 2001). Chilling injury, which contributes to the extensive damage that occurs during the process of cryopreservation, may occur because of slow changes in membrane properties and integrity (Martino *et al.*, 1996; Ghetler *et al.*, 2005). The cooling rate is a key factor in determining chilling resistance since it controls the duration of sample exposure to the temperatures within the 'dangerous zone' (Mazur *et al.*,1992). Previous results of blastocyst cryopreservation have been too inconsistent (Zhu *et al.*, 1993; Liebermann *et al.*, 2004) for routine use in IVF laboratories, emphasizing the difficulty of cryopreserving human blastocysts. This may be related to the high sensitivity of blastocysts to subphysiological temperatures during cooling (Stehlik *et al.*, 2005). Moreover, small volumes of cryoprotectant used for cryopreservation by direct falling of the blastocyst in to liquid nitrogen greatly increase the cooling and warming rate. Dropping the minimum volume, as small as 1 to 2 μl of embryo-containing solution directly into the LN_2 without the aid of any containers or carriers can minimize the

vapor formation and maximize heat exchange between sample and LN_2, thus to maximize the cooling rate (Huang *et al.*, 2006). The efficacy of vitrification in small volumes is demonstrated by good survival rates of early human blastocysts using the cryotop, the cryoloop (Reed *et al.*, 2002; Mukaida *et al.*, 2003) electron microscope grids (Choi *et al.*, 2000; Son *et al.*, 2003), glass micropipette(Cho *et al.*, 2002a) and the hemi-straw (Vanderzwalmen *et al.*, 2003).

The nuclear evaluation of mammalian blastocysts is a valid indicator of its viability (Papaioannou *et al.*, 1998; Hardy, 1999). Total cell number of blastocyst after 24 hr post-thaw IVC was similar between non-punctured and punctured groups. However, apoptotic cell number was higher in punctured than that in non-punctured groups. Presence of apoptotic cells in the blastocysts is a normal physiological phenomenon and it has been reported that as high as 91~100% of bovine blastocyst irrespective of origin (*in vivo* or *in vitro*) containing at least one apoptotic cell (Park *et al.*, 2006). Apoptosis is a highly conserved and regulated program that initiates cells death under a variety of internal and external controls (Dhali *et al.*, 2007) is an indicator of inadequate or suboptimal developmental conditions and stress (Betts *et al.*, 2001), and of embryo freezing damage (Park *et al.*, 2006). Apoptosis eliminate defective cells by accumulating genetic damage (Jurisicova *et al.*, 1998; Yoon *et al.*, 2001). These data collectively suggest that reduction of blastocoele fluid before vitrification will enhance post thaw blastocyst quality.

Hatching of blastocyst is a pre-requisite for successful implantation (Modlinski, 1970). In human, implantation and pregnancy rates are higher when hatched blastocysts transferred in to the patients than non-hatching blastocysts (Yoon *et al.*, 2001). Hatching rates were higher in punctured group than non-punctured group, suggesting high pregnancy rates following transfer of punctured frozen-thawed blastocysts. If the membrane of blastoceole is disrupted by AS, which can be assisted the hatching process.

In summary, *in vitro* Hanwoo blastocysts can be vitrified after removal of blastocoele fluid maintaining high post thaw embryo quality which may enhance implantation and pregnancy rates.

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REFERENCES

Andrabi SMH. 2007. Fundamental principles of cryopreservation of *Bos taurus* and *Bos indicus* bull spermatozoa. International Journal of Agriculture & Biology 9:367-369.

Arav A, Pearl M and Zeron Y. 2000. Does membrane lipid profile explain chilling sensitivity and membrane lipid phase transition of spermatozoa and oocytes. Cryo. Letters 21: 179-186.

Betts DH and King WA. 2001. Genetic regulation of embryo death and senescence. Theriogenology 55:171-191.

Cho SK, Cho SG, Bae IH, Park CS and Kong IK. 2002a. Improvement in post-thaw viability of *in vitro*-produced bovine blastocysts vitrified by glass micropipette (GMP). Anim. Reprod. Sci. 73:151-158.

Cho HJ, Son WY, Yoon SH, Lee SW and Lim JH. 2002b. An improved protocol for dilution of cryoprotectants from vitrified human blastocysts. Hum. Reprod. 17:2419-2422.

Choi DH, Chung HM, Lim JM, Ko JJ, Yoon TK and Cha KY. 2000. Pregnancy and delivery of healthy infants developed from vitrified blastocysts in an IVF-ET program. Fertil. Steril. 74:838-839.

Desai N, Szeptycki J, Scott M, AbdelHafez FF and Goldfarb J. 2008. Artificial collapse of blastocysts before vitrification: mechanical vs. laser technique and effect on survival, cell number, and cell death in early and expanded blastocysts. Cell Preservation Techonology 6:181-190.

Dhali A, Anchamparuthy VM, Butler SP, Pearson RE, Mullarky IK and Gwazdauskas FC. 2007. Gene expression and development of mouse zygotes following droplet vitrification. Theriogenology 68:1292-1298.

Dinnyes A, Dai Y, Jiang S and Yang X. 2000. High developmental rates of vitrified bovine oocytes following parthenogenetic activation, *in vitro* fertilization, and somatic cell nuclear transfer. Biol. Reprod. 63:513-518.

Foote RH and Parks JE. 1993. Factors affecting preservation and fertility of bull sperm: A brief review. Reprod. Fertil. Dev. 5:665-673.

Ghetler Y, Yavin S, Shalgi R and Arav A. 2005. The effect of chilling on membrane lipid phase transition in human oocytes and zygotes. Hum. Reprod. 20:3385-3389.

Hardy K. 1999. Apoptosis in the human embryo. Rev. Reprod. 4:125-134.

Hiraoka K, Hiraoka K, Kinutani M and Kinutani K. 2004. Blastocoele collapse by micropipetting prior to vitrification gives excellent survival and pregnancy outcomes for human day 5 and 6 expanded blastocysts. Hum. Reprod. 19:2884-2888.

Huang JC, Lin HH, Wu JS, Tang PH, Wang DG and Liu DG. 2006. Vitrification of caprine embryos in microdrops. Symposium COA/INRA Scientific Cooperation in Agriculture 7:47-58.

Jeong WJ, Cho SJ, Lee HS, Deb GK, Lee YS, Kwon TH and Kong IK. 2009. Effect of cytoplasmic lipid content on *in vitro* developmental efficiency of bovine IVP embryos. Theriogenology 72:584-589.

Jurisicova A, Latham KE, Casper RF, Casper RF and Varmuza SL. 1998. Expression and regulation of genes associated with cell death during murine preimplantation embryo development. Mol. Reprod. Dev. 51:243-253.

Kasai M, Zhu SE, Pedro PB, Nakamura K, Sakurai T and Edashige K. 1996. Fracture damage of embryos and its prevention during vitrification and warming. Cryobiology 33:459- 464.

Kuwayama M, Hamano S and Nagai T. 1992. Vitrification of bovine blastocysts obtained by *in vitro* culture of oocytes matured and fertilized *in vitro*. J. Reprod. Fertil. 96:187-193.

Liebermann J and Tucker MJ. 2004. Vitrifying and warming of human oocytes, embryos, and blastocysts: Vitrification procedures as an alternative to conventional cryopreservation. Methods Mol. Biol. 254:345-364.

Martino A, Pollard JW and Leibo SP. 1996. Effect of chilling bovine oocytes on their developmental competence. Mol. Reprod. Dev. 45:503-512.

Mazur P, Cole KW, Hall JW, Schreuders PD and Mahowald AP. 1992. Cryobiological preservation of Drosophila embryos. Science 258:1932-1935.

Modlinski JA. 1970. The role of the zona pellucida in the development of mouse eggs *in vivo*. J. Embryol. Exp. Morphol. 23:539-547.

Mukaida T, Nakamura S, Tomiyama T, Wada S, Oka C, Kasai M and Takahashi K. 2003. Vitrification of human blastocysts using cryoloops: Clinical outcome of 223 cycles. Hum. Reprod. 18:384-391.

Nedambale TL, Du F, Yang X and Tian XC. 2006. Higher survival rate of vitrified and thawed *in vitro* produced bovine blastocysts following culture in defined medium supplemented with beta-mercaptoethanol. Anim. Reprod. Sci. 93: 61-75.

Papaioannou VE and Ebert KM. 1988. The preimplantation pig embryo: Cell number and allocation to trophectoderm and inner cell mass of the blastocyst *in vivo* and *in vitro*. Development 102:793-803.

Park SY, Kim EY, Cui XS, Tae JC, Lee WD, Kim NH, Park SP and Lim JH. 2006. Increase in DNA fragmentation and apoptosis-related gene expression in frozen-thawed bovine blastocysts. Zygote 14:125-131.

Reed ML, Lane M, Gardner DK, Jensen NL and Thompson J. 2002. Vitrification of humanblastocysts using the cryoloop method: Successful clinical application and birth of offspring. Journal of Assisted Reproduction and Genetics 19:304-306.

Son W, Yoon S, Yoon H, Lee S and Lim J. 2003. Pregnancy outcome following transfer of human blastocysts vitrified on electron microscopy grids after induced collapse of the blastocoele. Hum. Reprod. 18:137-139.

Stehlik E, Stehlik J, Katayama KP, Kuwayama M, Jambor V, Brohammer R and Kato O. 2005. Vitrification demonstrates significant improvement versus slow freezing of human blastocysts. Reprod. Biomed. Online.11:53-57.

Yoon HJ, Yoon SH, Son WY, Im KS and Lim JH. 2001. High implantation and pregnancy rates with transfer of human hatching day 6 blastocysts. Fertil. Steril. 75:832-833.

Vanderzwalmen P, Bertin G, Debauche C, Standaert V, van Roosendaal E, Vandervorst M, Bollen N, Zech H, Mukaida T, Takahashi K and Schoysman R. 2002. Births after vitrification at morula and blastocyst stages: Effect of artificial reduction of the blastocoelic cavity before vitrification. Hum. Reprod. 17:744-751.

Vanderzwalmen P, Bertin G, Debauche C, Standaert V, Bollen N, van Roosendaal E, Vandervorst M, Schoysman R and Zech N. 2003. Vitrification of human blastocysts with the hemi- straw carrier: Application of assisted hatching after thawing. Hum. Reprod. 18:1504-1511.

Zeron Y, Ocheretny A, Kedar O, Borochov A, Sklan D and Arav A. 2001. Seasonal changes in bovine fertility: Relation to developmental competence of oocytes, membrane properties and fatty acid composition of follicles. Reproduction 121: 447-454.

Zhu SE, Kasai M, Otoge H, Sakurai T and Machida T. 1993. Cryopreservation of expanded mouse blastocysts by vitrification in ethylene glycol-based solutions. J. Reprod. Fertil. 98: 139-145.

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