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Cryopreservation of Bovine IVM/IVF/IVC Blastocysts by Vitrification

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체외성숙, 체외수정 및 체외배양에서 생산된 소 배반포기배의 초자화 동결

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= 국문초록 =

본 연구는 체외생산된 소 배반포기배를 발달 단계 및 배양일에 따라 구별하여 초자화 동결 및 융해하였을 때, 그 발달능을 유지하는지 확인하고자 실시하였다. 체외수정 후 8일간 배양된 배반포기배는 20% ethylene glycol에 3분 동안 평형시키고, EFS40 (40% ethylene glycol, 18% ficoll, 0.3 M sucrose 그리고 10% FBS가 함유된 mDPBS) 동결액에 30초 동안 노출한 후, 액체질소에 침지하여 초자화 동결되었다. 체외 생존 여부는 융해 24시간 및 48시간에 재팽창 및 탈출 또는 완전탈출로써 평가하였다. 그 결과를 요약하면 다음과 같다. 1) 체외수정 후 8일간 배양하였을 때, 난할된 배의 배반포기배로의 발달율은 41.0%였다 (초기; 7.6%, 팽창; 22.9%, 탈출; 4.6%, 완전탈출; 5.9%). 2) 배반포기배를 동결액에 노출 또는 초자화 동결하였을 때, 초자화 동결된 배반포기배의 재팽창율 (73.3%)은 대조군 및 동결액에 노출된 경우 (100, 97.0%) 보다 낮았다 ($p<0.05$). 그러나 융해 48시간 후 탈출 또는 완전탈출 배반포기배 형성율은 초자화 동결된 경우 (66.7, 46.7%)와 노출된 경우 (66.7, 39.4%)는 유의한 차이를 나타내지 않았으나, 대조군 (100, 100%)과는 차이를 보였다 ($p<0.01$). 그러나, 완전탈출까지 발달한 배반포기배의 총 세포수를 조사하였을 때, 각 처리군간의 유의한 차이는 없었다. 3) 배반포기배의 발달 단계에 따른 체외 생존율을 비교하였을 때, 재팽창율은 실험군간에 유의한 차이를 보이지 않았다 (64.5~75.6%). 그러나 융해 48시간 후, 탈출 또는 완전탈출로써 평가된 초기 배반포기배의 발달율 (25.8, 9.7%)은 팽창 (69.7, 39.4%) 및 탈출 배반포기배 (53.3, 43.3%)의 발달율보다 낮게 나타났다 ($p<0.05$). 4) 또한, 배양 7, 8 그리고 9일의 팽창 배반포기배를 초자화 동결하였을 때, 8일 및 9일간 배양된 배반포기배의 재팽창율은 7일간 배양된 경우보다 낮게 나타났다 (7일; 93.9%, 8일; 75.8%, 9일; 87.5%) ($p<0.05$). 그러나 완전탈출 배반포기배로의 발달율에서는 처리군간에 유의한 차이를 보이지 않았다 (7일; 36.4%, 8일; 36.4%, 9일; 31.3%). 이러한 결과는 EFS40을 이용한 2단계 초자화 동결 방법이 체외생산된 팽창 및 탈출 배반포기배의 동결에 유용하게 이용될 수 있음을 시사한다.

Key Words: Bovine, Vitrification, EFS40, Developmental stage, Embryo age

INTRODUCTION

The cryopreservation of mammalian embryos has become an integral part of methods to control animal reproduction (Bautista and Kanagawa, 1998). As an innovative method for embryo cryopreservation, vitrification not only reduced the cooling stage duration to a minimum, but also eliminated any injuries caused

by extracellular ice which is a major cause of cell injury (Kasai, 1997). Since the first successful vitrification of mammalian embryos was achieved by Rall and Fahy (1985), many researchers have focused on vitrification of different developmental stages in various species and subsequent production of offspring (Kim *et al.*, 1997; van Wagtendonk-de Leeuw *et al.*, 1997; Martinez and Matkovic, 1998). And after 1990, the cryoprotectant based on ethylene glycol (EG) which permeates the cell rapidly and has low toxicity is widely used. Also, the EG in combination with macromolecules and sugars, EFS has been used successfully for the vitrification of mouse morula and blastocysts (Kasai *et al.*, 1990; Kim *et al.*, 1997), rabbit morula (Kasai *et al.*, 1992), bovine blastocysts (Tachikawa *et al.*, 1993) and ovine morula (Martinez and Matkovic, 1998).

Survival of frozen-thawed *in vitro* produced embryos has been reported to be affected by embryo age, stage of embryonic development, embryo quality, cryoprotectant, pH of the freezing medium, freezing process and culture system (medium and gaseous atmosphere) in which the embryos are produced (Leibo and Loskutoff, 1993; Hasler *et al.*, 1997).

The objective of this study was to examine the effect of developmental stage of *in vitro* produced bovine blastocysts vitrified by EFS.

MATERIALS AND METHODS

1. Production of bovine *in vitro* maturation, fertilization and culture (IVM/IVF/IVC) blastocysts

The method used for production of bovine embryos is described in detail by Park *et al.* (1995). Bovine ovaries were transported from a slaughterhouse to the laboratory in saline (32±2℃) in container. Cumulus oocytes complexes (COCs) were collected from visible follicles (2~6 mm) of ovaries. The COCs were washed three times with TL-HEPES (low carbonate TALP) medium containing 1 mg/ml of bovine serum albumin (BSA; Fraction V, Sigma). The basic medium for maturation was tissue culture medium (TCM-199). The additives were fetal bovine serum (FBS; 10%), sodium pyruvate (0.2 mM), FSH (1 μg/ml), estradiol-17β (1 μg/ml) and gentamycin (25 μg/ml). Ten COCs were placed in each maturation drop (50 μl), and then cultured for 22~24 h at 39℃, 5% CO_2 incubator.

For *in vitro* fertilization (IVF), highly motile spermatozoa were recovered from frozen-thawed bull semen separated on a discontinuous percoll column and resuspended with Sp-TALP at a concentration of 2.5×10^7 cells/ml. After 22~24 h *in vitro* maturation, the COCs were washed three times with Sp-TALP, and transferred into 44 μl fertilization drops. Ten COCs were moved to each fertilization drop, followed by 2 μl of motile sperm (5×10^4 cells/50 μl drop), 2 μl of heparin (2 μg/ml) and 2 μl of PHE (18.2 μM Penicillamine, 9.1 μM Hypotaurine and 1.8 μM Epinephrine) stock.

The cleaved embryos were selected at day 2 after IVF and then cultured in 50 μl of mCR1-FAF medium (which was supplemented with 3 mg/ml of fatty acid free BSA) under mineral oil. At day 4, the embryos were transferred to mCR1-FBS medium (which was supplemented with 10% FBS). For experiments, the expanded blastocysts produced at day 7, 8 and 9 after IVF and the early, expanded and hatching blastocysts obtained at day 8 (Fig. 1A) were used.

2. Freezing and thawing

The vitrification solution used for experiment was based on that described by Kasai *et al.* (1990). The vitrification solution, EFS40, consisted of 40% (v/v) ethylene glycol (EG, Sigma), 18% (w/v) ficoll 70 (Sigma) and 0.3 M sucrose (Sigma) and 10% FBS dissolved in modified Dulbecco's phosphate-buffered saline (mDPBS, Gibco BRL). Blastocysts were equilibrated in 20% EG for 3 min. and exposed to EFS40 (Fig. 1B) and loaded into 0.25 ml straw for 30 sec. at 25℃. The plug end of each

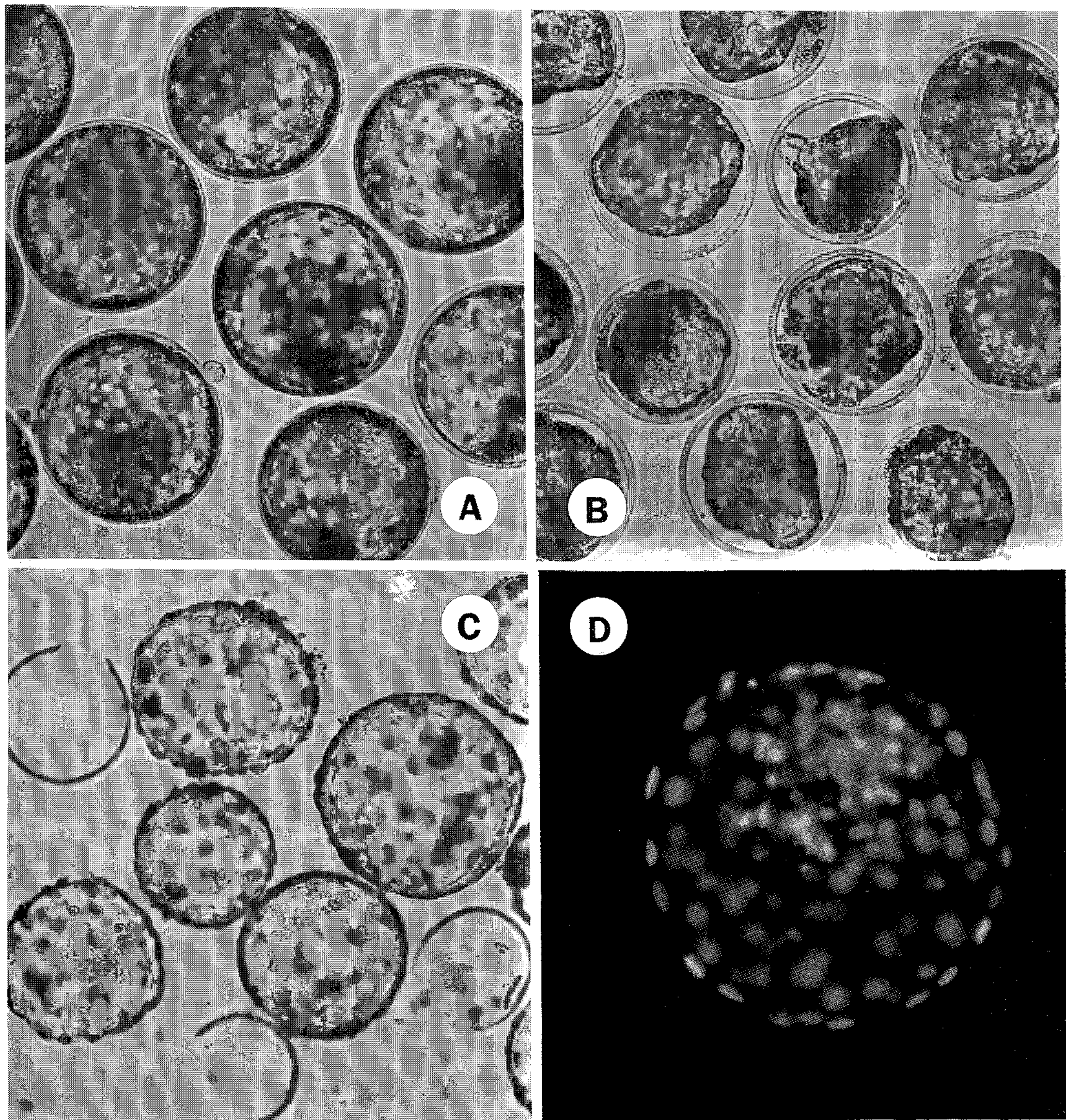

Fig. 1. A series of course from vitrification to survival after thawing. **A.** Expanded blastocysts on day 8 (×150), **B.** Expanded blastocysts exposed to 20% EG (×150), **C.** Developed blastocysts at 48 h after thawing (×100), **D.** Hoechst stained hatched blastocyst at 48 h after thawing (total cell number; 152) (×300).

straws was treated with powder and heat sealed. Then the straw was slowly immersed into liquid nitrogen (LN_2) for 10 sec. About 5~9 embryos were loaded in each straw.

After a few days or months of storage in LN_2, the straws were placed in air for 3 sec. and then warmed rapidly in water bath at 25℃. The contents of each straw were expelled into dish containing 0.8 ml of mDPBS containing 0.3 M sucrose and 10% FBS (S-DPBS) by cutting the cotton plug and then the recovered embryos were put into fresh 0.3 M S-DPBS for 5 min. The embryos were transferred to mDPBS with 10% FBS for 5 min. Blastocysts were cultured in a 10 μl droplet of co-culture medium (cumulus cell monolayer + mCR1-FBS).

3. Assessment of survival

Survival rate after thawing was morphologically assessed by examining re-expansion of blastocoele at 24 h of *in vitro* culture and development to hatching or hatched stage at 48 h of culture (Fig. 1C).

4. Total cell count

To compare the effect of vitrification solution and freezing to the cell number of blastocyst, total cell number of hatched blastocysts (HBs) after final observation was counted. The HBs were fixed with 2% formalin solution for 3 min. and stained with bisbenzimide solution (No. 33342, 2.5 μg/ml, Sigma). Observation was carried out under ultra violet filter incorporated fluorescent microscope on day 1 after making sample (Fig. 1D).

5. Experimental design

1) Toxicity and freezing effect of vitrification using EFS40

To investigate the toxicity of vitrification solution and freezing effect, the expanded blastocysts on day 8 were divided at random into 3 groups (control, exposed and vitrified). The exposed group was put through the same procedure as vitrification except being plunged into LN_2. And the vitrified group was treated as described above.

2) *In vitro* survival of bovine blastocysts vitrified at different developmental stage

To investigate the effect of vitrification using EFS40 according to different developmental stage, the embryos on day 8 were divided into early, expanded and hatching blastocysts by morphological characteristics (Kim *et al.*, 1996). And the two-step vitrification and thawing methods were carried out as described a bove.

3) *In vitro* survival of bovine blastocyst vitrified at different embryo age

To examine the viability of embryo accor ding to embryo ages, the expanded blastocyst developed on day 7, 8 and 9 of culture afte IVF were vitrified and thawed, respectively.

6. Statistical analysis

The survival rates after each treatment wer compared using Chi-square tests using SAS in stitute software. And the difference in numbe of cells between the HBs of each treated grou was compared using the Student's t-test.

RESULTS

1. Production of bovine IVM/IVF/IVC blastocysts

The development rates of *in vitro* produce embryos were shown in Table 1. The cleavage rate on day 2 after IVF was 87.3% and blastocyst formation rate on day 8 was 41.0% Also, when the blastocysts on day 8 were classified to early, expanded, hatching and hatched, their development rates were 7.6, 22.9, 4.6 and 5.9%, respectively.

2. Toxicity and freezing effect of vitrification using EFS40

The survival rates *in vitro* of day 8 bovine blastocyst exposed or vitrified in EFS40 were presented in Table 2. When the embryos were exposed or vitrified to the freezing solution, the re-expansion rates of control, exposed and vitrified embryos were 100, 97.0 and 73.3%, respectively. The re-expansion rate of vitrified embryos was lower than that of control and ex-

Table 1. Developmental capacity of bovine IVM/IVF/IVC embryos on day 8 (r=6)

No. of embryos cultured	No. (%) of cleaved (≥2-cell)	No. (%) of blastocysts developed into				
		Total	Early	Expanded	Hatching	Hatched
526	459 (87.3)	188 (41.0)	35 (7.6)	105 (22.9)	21 (4.6)	27 (5.9)

Table 2. Survival *in vitro* of day 8 bovine blastocysts vitrified in EFS40

Treatment	No. of blastocysts*	No. (%) of blastocysts†						Cell number (Mean±S.E.)
		Vit.	Recov.	Survived				
				24 h		48 h		
				≥ Re-ex	≥ Hg	≥ Hg	Hd	
Control	31	–	–	31 (100)[a]	29 (93.5)[a]	31 (100)[a]	31 (100)[a]	142.7±16.0
Exposed	33	–	33 (100)	32 (97.0)[b]	15 (45.5)[b]	22 (66.7)[b]	13 (39.4)[b]	120.1±8.1
Vitrified	31	31	30 (96.8)	22 (73.3)[c]	14 (46.7)[b]	20 (66.7)[b]	14 (46.7)[b]	118.9±12.0

*The blastocysts of expanded stage were examined.
† Vit.; Vitrified, Recov.; Recovered, Re-ex; Re-expansion, Hg; Hatching blastocysts, Hd; Hatched blastocysts
a,b,c Superscripts were significantly different within the same column (p<0.01).

Table 3. Survival *in vitro* of day 8 bovine blastocysts vitrified in EFS40 by different developmental stages

Blastocyst stage	No. of blastocysts	No. (%) of blastocysts†					
		Vit.	Recov.	Survived			
				24 h		48 h	
				≥ Re-ex	≥ Hg	≥ Hg	Hd
Early	31	31	31 (100)	20 (64.5)	4 (12.9)[a]	8 (25.8)[c]	3 (9.7)[a]
Expanded	33	33	33 (100)	25 (75.6)	16 (48.5)[b]	23 (69.7)[d]	13 (39.4)[b]
Hatching	31	31	30 (96.8)	21 (70.0)	21 (70.0)[b]	16 (53.3)[d]	13 (43.3)[b]

a,b,c,d Superscripts were significantly different within the same column (a, b; p<0.01, c, d; p<0.05).
† Vit.; Vitrified, Recov.; Recovered, Re-ex; Re-expansion, Hg; Hatching blastocysts, Hd; Hatched blastocysts

posed embryos (p<0.01). But the formation rates of hatching or HBs of vitrified embryos (66.7, 46.7%) at 48 h after thawing were similar to those of exposed embryos (66.7, 39.4%) but not control (100, 100%) (p<0.01).

Total cell number of developed HBs of control group was 142.7±16.0 and that of exposed and vitrified group was 120.1±8.1 and 118.9±12.0, respectively. There were not significantly different among the treatment groups.

3. *In vitro* survival of bovine blastocysts vitrified at different developmental stage

As shown in Table 3, when the embryo survival rates by different developmental stage were examined, the re-expansion rates of early, expanded and hatching blastocyst were 64.5, 75.6 and 70.0%, respectively. The re-expansion rate was not different among the groups. After warming 48 h, the hatching or hatched formation rate of early blastocysts was 25.8 or 9.7% and that of expanded and hatching blastocysts was 69.7 or 39.4% and 53.3 or 43.3%, respectively. The developmental rate of early blastocyst was significantly lower than that of expanded and hatching blastocysts (p<0.05).

4. *In vitro* survival of bovine blastocysts vitrified at different embryo age

In Table 4, when the expanded blastocysts on day 7, 8 and 9 were vitrified, the re-expansion rate of day 7 embryos was 93.9% and that of day 8 and 9 embryos was 75.8 and 87.5%. The re-expansion rates of day 8 and 9 embr-

Table 4. Survival *in vitro* of bovine blastocysts vitrified in EFS40 by different ages

Culture day	No. of blastocysts*	No. (%) of blastocysts†					
		Vit.	Recov.	Survived			
				24 h		48 h	
				≥ Re-ex	≥ Hg	≥ Hg	Hd
7	33	33	33 (100)	31 (93.9)[a]	11 (33.3)	24 (72.7)	12 (36.4)
8	34	34	33 (97.1)	25 (75.8)[b]	13 (39.4)	21 (63.6)	12 (36.4)
9	32	32	32 (100)	28 (87.5)[b]	9 (28.1)	18 (56.3)	10 (31.3)

*The blastocysts of expanded stage were examined.
† Vit.; Vitrified, Recov.; Recovered, Re-ex; Re-expansion, Hg; Hatching blastocysts, Hd; Hatched blastocysts
a,b Superscripts were significantly different within the same column ($p<0.05$).

yos were significantly lower than that of day 7 ($p<0.05$). However, the formation rates of hatching and hatched were 72.7 and 36.4% for day 7, 63.6 and 36.4% for day 8 and 56.3 and 31.3% for day 9 embryos, respectively. The developmental capacity was no difference among the groups.

DISCUSSION

In the vitrification of bovine expanded blastocysts, it was reported that two-step method using 20% EG and EFS40 was effective (Mahmoudzadeh *et al.*, 1995). However, in the present experiments, to confirm toxicity of vitrification solution and freezing effect, the re-expansion rate and developmental capacity of the exposed and vitrified embryos were lower than those of control. That is, it was appeared the toxicity to vitrification solution and the freezing effect. Generally, it is known that one essential factor in survival of frozen-thawed embryos is the permeation of a certain amount of the cryoprotectant into the embryo (Tachikawa *et al.*, 1993). Thus, to avoid the toxic action of the cryoprotectant, the duration of exposure should be adjusted. However, total cell number of developed hatched blastocysts of control, exposed and vitrified groups was not different. This findings showed that when *in vitro* produced bovine blastocysts were vitrified the two-step method, the survival rate of those was reduced than control, but the quality of frozen-thawed blastocyst was not bad (Saha *et al.*, 1996).

Possible factors producing differences in the survival rates of vitrified oocytes and embryos at different stages of development would be 1) chemical toxicity of the cryoprotectants, 2) osmotic shrinkage of the cell, 3) intracellular ice formation, 4) vitrification, 5) osmotic swelling of the cell during removal of the permeated cryoprotectant, 6) quality of embryos and 7) developmental stages of blastocysts (Han *et al.*, 1994; Martinez and Matkovic, 1998).

In the present study, the re-expansion or developmental rates of early blastocyst were lower than those of expanded and hatching blastocysts. Mahmoudzadeh *et al.* (1995) and Hasler *et al.* (1997) reported that the stage of embryonic development has influence on frozen-thawed survival rate. It is caused that early blastocysts stage of *in vitro* produced embryos has been shown to be more sensitive to chilling injury than expanded blastocysts (Pollard and Leibo, 1994). Also, it is known that blastocysts developed earlier have more cells and fewer chromosomal anomalies than those developed later (Zhu *et al.*, 1996). Thus, *in vitro* produced bovine embryos have been efficiently cryopreserved on day 7 rather than on day 8 or 9 (Zhu *et al.*, 1996). However, our results

showed that not only expanded blastocysts on day 7 but also those on day 8 or 9 could be efficiently cryopreserved. Hasler *et al.* (1997) reported that embryos at any given stage of development had highest survival rates when frozen at the earliest age the stage appeared. Also, Saha *et al.* (1996) reported that when day 7, 8 and 9 embryos were vitrified separately, the viability of those differed significantly. But, our result was similar to other report (Han *et al.*, 1994). It is denoted that bovine IVM/IVF/IVC system and vitrification and thawing methods in our laboratory are stable.

These results indicated that *in vitro* produced expanded or hatching blastocysts can be efficiently cryopreserved by the two-step vitrification method using EFS40.

SUMMARY

The objective of this study was to examine the effect of developmental stage and embryo age of *in vitro* produced bovine blastocysts after vitrification and thawing. *In vitro* cultured day 8 blastocysts after IVF were equilibrated 20% ethylene glycol (EG) for 3 min. and were vitrified using EFS40, which is consisted of 40% EG, 18% ficoll, 0.3 M sucrose and 10% FBS added in mDPBS for 30 sec. before being plunged into LN_2. Also, survival *in vitro* was assessed by re-expansion and hatching or hatched at 24 h and 48 h postwarming, respectively. The results obtained in these experiments were summarized as follows; 1) When the embryos were cultured for 8 day after IVF, 41.0% of the cleaved embryos developed to the blastocysts (early; 7.6%, expanded; 22.9%, hatching; 4.6% and hatched; 5.9%). 2) When the embryos were exposed or vitrified to the freezing solution, the re-expansion of vitrified embryos (73.3%) was significantly lower than that of control and exposed embryos (100, 97.0%) ($p<0.05$). But the formation rate of hatching or hatched blastocysts of vitrified embryos (66.7, 46.7%) at 48 h after thawing was similar to that of exposed embryos (66.7, 39.4%) but not control (100, 100%) ($p<0.01$). However, in the total cell numbers of those developed hatched blastocysts, there were not significantly different among the treatment groups. 3) When the embryo survival rates by different developmental stage were examined, the re-expansion was not different among the groups (64.5~75.6%). After warming 48 h, the hatching and hatched formation of early blastocysts (25.8, 9.7%) was significantly lower than those of expanded (69.7, 39.4%) and hatching blastocysts (53.3, 43.3%) ($p<0.05$). 4) In addition, when the expanded blastocysts at day 7, 8 and 9 were vitrified, the re-expansion of day 8 and 9 embryos was significantly lower than that of day 7 (day 7; 93.9%, day 8; 75.8% and day 9; 87.5%) ($p<0.05$). However, the rates of development to hatched blastocysts were no difference among the groups (day 7; 36.4%, day 8; 36.4% and day 9; 31.3%). These results suggested that *in vitro* produced expanded or hatching blastocysts can be efficiently cryopreserved by the two-step vitrification method using EFS40.

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