Original Article

In Vitro Development Potentiality of Expanded Bovine Blastocysts Subsequent to Cryotop Vitrification

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Abstract

Embryo cryopreservation is a promising area of study in the field of reproductive research. One of the issues that have recently arisen is the discovery that the growing ability of embryos after cryopreservation varies depending on the culture period of the embryo prior to its cryopreservation. Therefore, the present study was designed to explore the *in vitro* development potentiality of bovine embryos at day 7 and day 8, as well as their survivability and hatchability after Cryotop vitrification. The blastocyst rate at day 8 (28.1%) was higher than that of day 7 (19.1%). Grade 1 (G1) and grade 2 (G2) expanded blastocysts at day 7 and day 8 were vitrified by Cryotop device using 20% (v/v) DMSO, 20% (v/v) EG and 0.5M. Except for the G2 expanded blastocysts at day 8, we found that the survival rates of vitrified G1 and G2 expanded blastocysts were not significantly different from the control group in both day 7 and day 8. However, the day 7 vitrified embryos showed superior rates of hatchability than those of day 8. In a curve estimation of correlation regressions, the hatching rate of day 7 G1 expanded blastocysts at 48 h showed a strong correlation (R^2=0.914) with their survival rate. Therefore, we concluded that the day 7 culture period is the most suitable for vitrification of IVF derived blastocysts.

Keywords: blastocysts,bovine, *in vitro* fertilization, vitrification

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Introduction

Vitrification of oocytes and embryos (of different species of animals as well as humans) is an important field of research. Successful vitrification of mammalian embryos has numerous economic benefits, and can have a major impact on improving the efficiency of animal research, breeding and production. The first successful cryopreservation of bovine embryos was reported by Wilmut and Rowson (1973). Nowadays, the focus is on studying the vitrification of embryos using different devices and cryoprotective agents (CPAs) in order to increase their survival rate as well as the success rate of ET. High survival rates after bovine embryo vitrification have recently been achieved using different devices and techniques. For instance, electron microscopic (EM) grid (Steponkus et al., 1990; Martino et al., 1996; Cho et al., 2002), minimum drop size (MDS; Arav, 1992, Arav and Zeron, 1997; Yavin and Arav, 2001), Cryotop (Hamawaki et al., 1999; Kuwayama and Kato, 2000), Cryoloop (Lane et al., 1999), Hemi-straw (Vanderzwalmen et al., 2003), solid surface vitrification (SSV; Dinnyes et al., 2000), open pulled straw (OPS; Vajta et al., 1997, 1998), closed pulled straw (CPS; Chen et al., 2001), Rapid-i (Larman and Gardner, 2010) and hollow fiber (Matsunari et al., 2012) are some of the devices and procedures that have been used in animal oocyte and embryo vitrification.

The survival rate of embryos is markedly dependent on their exposure to cryoprotective agents (CPAs) prior to their immersion in liquid nitrogen (Inaba et al., 2011). On the other hand, using the Cryotop device to achieve minimum volume of vitrification solution (VS) is a novel vitrification method. The Cryotop method has been efficaciously used to cryopreserve bovine (Chian et al., 2004), bubaline (Gaspirini et al., 2007) and ovine oocytes (Succu et al., 2007), in addition to embryos in bovine and bubaline species (Laowtammathron et al., 2005). But the compositions and concentration of CPAs are also very important for successful vitrification.

Although successful cryopreservation depends, at least in part, on the modality of the freeze-thaw technique employed, embryo's developmental ages (in terms of "days") as well as its blastocyst stage are also important issues. The survival and hatching rate of vitrified embryos has been found to vary in relation to their developmental day/age and blastocyst stage at the time of their vitrification (Machatkova et al., 2006). One possible reason for this may be that later stage (and thus more expanded) blastocysts are more sensitive to cryopreservatives than early stage blastocysts because detrimental ice crystal formation may occur in the larger, later stage, blastocoel cavity due to insufficient dehydration (Stachecki et al., 2008). Similarly, the post-warming survival rate as well as the hatching rate might also be influenced by the day of development of embryos and their expanded blastocyst stage at the time of their vitrification. Vitrification of blastocysts involves their exposure to highly concentrated CPAs to prevent ice crystal formation (Rall and Fahy, 1985; Kim et al., 2012). As most CPAs are embryo-toxic, this can result in severe embryonic damage (Katkov, 2007). As a result, the concentration and duration of exposure to CPAs obviously are important issues for successful vitrification (Kim et al., 2012). However, to our knowledge, there are no specific reports and/or published data regarding the vitrification and post-warming of bovine expanded blastocysts at diverse culture periods and their co-relationship with hatchability rates. Therefore, the aim of this study was to evaluate the extent to which the chronology of development affects the cryotolerance of *in vitro* derived embryos vitrified using the Cryotop method. In particular, this was evaluated by assessing the developmental speed of embryos in different days of culture, their survival rates after vitrification and re-development into different grades and days of expanded blastocysts and the correlation between the post-warming survival rate and the hatchability rate of the redeveloped blastocysts.

Materials and Methods

Chemicals and media: All reagents were purchased from Sigma Chemical Company (St. Louis, MO, USA) unless otherwise stated. The medium used for IVM was TCM199 supplemented with 10% (w/v) fetal bovine serum (FBS; Gibco BRL, Grand Island, NY, USA), 0.02 AU/ml FSH (Antrin, Kyoritsu Seiyaku, Tokyo, Japan), 50 IU/ml hCG (Chorulon, Intervet, Boxmeer, Netherlands) and 1 μg/ml estradiol-17β. The medium for embryo culture was modified synthetic oviduct fluid supplemented with amino acids and 0.3% (w/v) fatty acid-free BSA (mSOF; Gardner et al., 1994).

Oocyte collection and in vitro maturation (IVM): Bovine ovaries were obtained from slaughter houses and kept at room temperature in 0.9% (w/v) NaCl for up to 4 h during their transport to the laboratory. Cumulus-oocyte complexes (COCs) were collected from follicles 2 to 8 mm in diameter using an 18-gauge needle connected to a 10 ml syringe. A group of 20 COCs were cultured in 100 μl droplets of IVM medium covered with mineral oil in a humidified atmosphere of 5% CO_2 in air at 38.5°C for 22-23 h.

In vitro fertilization (IVF) and culture (IVC): After IVM, cumulus cells were partially denuded from oocytes by gentle pipetting with a fine glass pipette with 0.1% hyarulonidase. After that a group of 10 oocytes were washed 3-4 times in Tyrode's Albumin Lactate Pyruvate (TALP) medium supplemented with 1 mmol caffeine, 100 μg/ml heparin, 20 mmol/l penecilamine, 10 mmol/l hypotaurine and 20 mmol/l ephinephrine. The sperm were prepared by swim up technique. One straw (0.25-ml straws, 25 $\times 10^6$ sperms/straw) was thawed at 39°C for 30 seconds. One hundred μl of thawed semen was placed into the bottom of a snapped tube containing 2 ml of TALP and kept at a 45° angle in a humidified atmosphere of 5% CO_2 in air at 38.5°C for 30 min. Then, supernatum was collected in a 15 ml conical tube and centrifuged at 500×g for 5 min. After centrifuging, the supernatum was discarded and 3 ml TALP was added for washing. After that it was centrifuged and sperm concentration was calculated by using a hemocytometer and adjusted

to 2×10^6 /ml (Seneda et al., 2001). Finally, 10 oocytes were kept in each 100 µl drop of sperm suspension and sperm-oocytes were co-incubated in a humidified atmosphere of 5% CO_2 in air at 38.5°C for 13-14 h. Then, presumptive zygotes were further cultured in mSOFaa medium (20 zygotes/100 µl) under a humidified atmosphere of 5% CO_2, 5% O_2 and 90% N_2 at 38.5°C for 48 h. At day 2, cleavage rates were recorded using the day of IVF as day 0. Thereafter, embryos at the 8-cell stage were selected and co-cultured with bovine oviduct epithelial cells in mSOFaa medium under a humidified atmosphere of 5% CO_2 in air at 38.5°C, as reported previously (Parnpai et al., 1999) for 6 days. Half of the medium was replaced with fresh medium during the embryos development and their stages and growth rates were recorded. Development to the blastocyst stage was noted on day 7 and day 8 (the day of IVF was considered as day 0).

Grading and vitrification of blastocysts: The blastocysts were graded according to the manual of The International Embryo Transfer Society (Robertson and Nelson, 1998), as to their development and quality of their inner cell mass (ICM) and trophectoderm (TE). First, the blastocyst was graded into categories of blastocyst (the volume of blastocoel is more than 50% of the total volume) and expanded blastocyst (Exp-BL; the blastocoel occupies the whole volume inside the zona pellucida (ZP) and has started expansion, leading to a thinning of the ZP). After this the blastocyst and expanded blastocyst stages were divided into G1 (ICM contains many small compacted cells and TE appears clearly made of many small cells tightly attached and forming a single layer epithelium) and G2 (cells in ICM are still separated and poorly compacted and TE is identifiable and contains few cells). The vitrification and warming of embryos by the Cryotop method was done according to the technique described by Liang et al. (2011). Briefly, groups of 1-3 Exp-BLs were washed in TCM199-Hepes + 20% (w/v) FBS (base medium; BM) before being placed in BM containing 10% (v/v) DMSO and 10% (v/v) EG (equilibration solution) for 1 min, and then exposed to BM containing 20% (v/v) DMSO, 20% (v/v) EG and 0.5 M sucrose (vitrification solution, VS) for 30 sec at 22 to 24°C. Finally within 30 sec, embryos with about 2 µl of VS were loaded onto Cryotops (Kitazato BioPharma, Fujinimiya, Japan) and plunged directly into liquid nitrogen (LN_2). The vitrified Cryotops were warmed by immersing them directly into 2 ml of 0.5 M sucrose in BM at 38.5°C on a warm plate for 5 min, washed 5 times, and then put in BM for 5 min and washed again 5 times. Finally, the warmed blastocysts were washed two times in an mSOFaa medium and transferred to a culture dish and cultured for another 2 days under a humidified atmosphere of 5% CO_2 in air at 38.5°C.

Experimental design

Growing ability of embryos at day 7 and day 8: Numbers of G1 and G2 blastocysts, and expanded blastocysts, were noted at day 7 and day 8 to record developmental speed of the blastocysts. A count was also done of a total number of developed blastocysts at day 7 as well as at day 8.

Assessment of developmental speed of day 7 and day 8 Exp-BLs subsequent to Cryotop vitrification: The grade 1 and 2 expanded blastocyst at day 7 and day 8 were vitrified by the Cryotop method, then warmed and checked. Their survivability was assessed after culture for 24 h. The re-expansion of the embryo within 24 h was used as an indicator of blastocyst survival. The hatching (Hing) and hatched blastocysts (Hed-BLs) were documented at 24 h and 48 h after warming and culture. The survivability, Hing as well as Hed-BLs of G1 and G2 stage Exp-BLs were noted and compared with the fresh control group.

Estimation of strength of correlation between embryo survival rate and hatching rate: The survival and hatching rate of G1 and G2 Exp-BLs at day 7 and day 8 periods were calculated and compared to estimate the correlation regressions and strength of influence between them 48 h post-warming culture.

Statistical analysis: The data of embryo development as well as their survival and re-developmental rates after vitrification and warming were expressed as percentages (%). The data was analyzed by using ANOVA. When ANOVA revealed a significant effect, the experimental groups were compared by Turkey method. Chi-square test was applied when qualitative evaluation was considered. The estimations of the correlation (R square value) between the embryo survival rate and the hatching rate were done by curve estimation of regressions (Menard, 2000) using SPSS® Software (version 16.0, SPSS Inc., Chicago, IL, USA). Differences were considered significant at a level of $p<0.01$ and $p<0.05$.

Results

Growing ability of embryos at day 7 and day 8: As shown in Fig.1, the average blastocyst production rate at day 8 (28.2%) was higher than that of day 7 (19.1%). The developmental frequency of G1 and G2 blastocysts at day 7 (5.1 and 3.3%, respectively) was greater than at day 8 (2.6 and 3.0%, respectively). In relation to Exp-BLs, the rate of development of both G1 and G2 Exp-BLs at day 8 (11.4 and 11.0%, respectively) was also greater compared to day 7 (6.4 and 4.6%, respectively). The rates of blastocyst development in more advanced stages were found to be dependent on the duration of culture.

Assessment of progressive developmental potentiality of expanded blastocysts subsequent to Cryotop vitrification: The survival rates of G1 Exp-BLs in both day 7 (97.1%) and day 8 (85.7%) culture periods were not significantly ($p>0.05$) higher than those of the fresh blastocysts (100 and 100%) as shown in Table 1. After 24-h culture, both the fresh G1 and G2 Exp-BLs showed a significantly ($p<0.05$) higher tendency to develop into a Hing-BL stage than those of the vitrified groups at both day 7 and day 8. The hatchability rate of G1 Exp-BLs in both periods was lower than that of the fresh control group, but did not show any significant variance at 24 h or 48 h post-warming culture. On the other hand, the vitrified G2 Exp-BLs revealed a significantly ($p<0.05$) lower Hed-BL rate at 24 h and

48 h post-warming culture compared with that of the fresh control vitrified in both day 7 and day 8.

Estimation of strength of correlation between blastocyst survival rate and hatching rate: We found that the hatching rate of embryos at 48 h post-warming culture showed a strong correlation with its corresponding survival rate as shown in Figs. 2 and 3. However, the G1 blastocysts at day 7 showed a significantly (p<0.01) higher correlation between hatchability and their survival rate.

Discussion

In this study, we demonstrated that the growth of good quality embryos at different culture periods was dissimilar. Currently, the study of vitrification techniques seems to be a primary focus among many embryologists. However, the success rate of vitrification, implantation subsequent to ET, as well as the pregnancies deriving from these procedures, are still subject to many variations and are open to question. All of these are primarily dependent on the day of culture period, the stage of the embryo, and the embryo's quality prior to vitrification. In this study we have proven which stage of bovine blastocysts is most favorable and adaptable for vitrification, as well the best culture period for vitrification in terms of the subsequent survivability and hatchability of the embryos involved.

Table 1 Post-warming survivability and development potentiality of G1 and G2 expanded blastocysts subsequent to Cryotop vitrification at day 7 and day 8

Day	Embryo types	Grade (G)	No. of embryos	Survival (%)	Hatching rate (%) at			
					24 h		48 h	
					Hing	Hed	Hing	Hed
D7	Fresh	G1	35	35/35 (100)[a]	13/35 (37.1)[a]	20/35 (57.1)[a]	2/35 (5.7)[a]	33/35 (94.3)[a]
		G2	33	33/33 (100)[a]	18/33 (54.5)[a]	9/33 (27.3)[b]	10/33 (30.3)[b]	23/33 (69.7)[a]
	Vitrified	G1	35	34/35 (97.1)[a]	6/35 (17.1)[b]	17/35 (48.6)[a]	3/35 (8.6)[a]	29/35 (82.8)[a]
		G2	31	21/31 (67.7)[a]	6/31 (19.4)[b]	5/31 (16.1)[c]	5/31 (16.1)[c]	12/31 (38.7)[b]
D8	Fresh	G1	34	34/34 (100)[a]	21/34 (61.8)[a]	13/34 (38.2)[b]	6/34 (17.6)[bc]	29/34 (85.3)[a]
		G2	36	36/36 (100)[a]	19/36 (52.8)[a]	8/36 (22.2)[bc]	15/36 (41.7)[d]	19/36 (52.8)[a]
	Vitrified	G1	28	24/28 (85.7)[a]	6/28 (21.4)[b]	6/28 (21.4)[bc]	2/28 (7.1)[a]	20/28 (71.4)[a]
		G2	26	13/26 (50)[b]	2/26 (3.8)[c]	2/26 (7.7)[d]	6/26 (23.1)[b]	4/26 (15.4)[c]

Different letters ([a, b, c, d]) within a column represent significant differences (P < 0.05).
Hing: Hatching, Hed: Hatched

In investigating good quality embryo development at different cultural periods we found that the developmental potentiality of G1 and G2 blastocysts was not significantly different between day 7 and day 8 culture periods, but that the rate of expanded blastocyst advancement at day 8 was higher than that of day 7. Our results partially or fully agreed with other studies in embryo development (Machatkova et al., 2005; Niemann et al., 1986). The rate of blastocyst development has been found to vary markedly according to the length of the culture period (Machatkova et al., 2005). Niemann et al. (1986) has stated that the frequency rate of good quality embryo development may vary in relation to its age. Recently, Sugimura et al. (2012) stated that the quality of blastocysts was also related to their hatchability after 48 h of additional post-warming culture of the expanded blastocysts.

In this study, we found that the survival rate of G1 Exp-BLs was significantly higher than that of G2 in both day 7 and day 8 culture periods; but the survivability of good quality embryos was not significantly different from the fresh group even between these vitrification periods. The survivability rates of good quality Exp-BLs in our study were similar to the rates in a study by Inaba et al. (2011; 94.3%), who

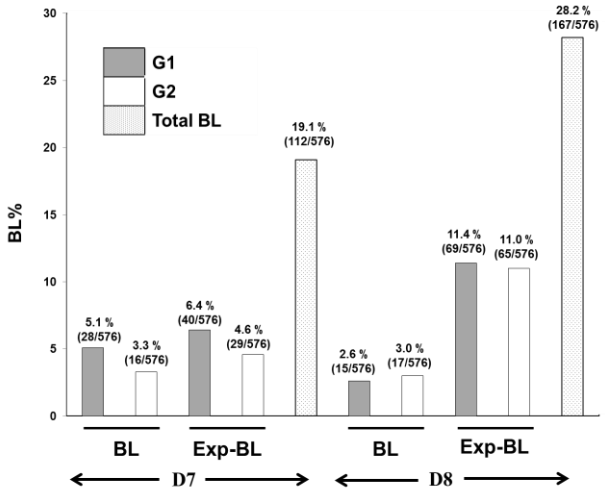

Figure 1 Growing ability of embryos at day 7 and day 8 culture periods
G1: Grade 1 blastocyst
G2: Grade 2 blastocyst
T BL: Total number of blastocysts
BL: Blastocyst
Exp-BL: Expanded blastocyst
D7: Data recorded at day 7
D8: Data recorded at day 8

used only good quality embryos for different vitrification techniques. In contrast, our vitrification and warming methods showed almost double the survivability rate than that of other studies that did not use only good quality embryos. Piereira et al. (2007) used a medium of PBS supplemented with 15% FCS and PBS with 15% (w/v) FCS and 5% (v/v) glycerol for 10 min and then finally transferred embryos to PBS supplemented with 10% (v/v) glycerol and 15% (w/v) FCS for another 10 min and had a survivability rate of 34.7%. Machatcova et al. (2006) used 10% (v/v) glycerol in TCM 199 medium with 10% (w/v) ECS, equilibrated embryos for 5 min at room temperature and found a survivability rate of 51.6%. Bruyere et al. (2012) used a different synthetic substitution (CRY03) of animal derived serum and found that it increased the embryo survival rate by inhibiting the fragility of the embryos. Fahning and Garcia (1992), Balasubramanian et al. (1998) and Machatkova et al. (2006) studied factors involved in the post thaw survival of *in vitro* embryos and demonstrated that the cryosurvival of an embryo depended on its morphology and developmental stage at the moment of freezing. Even though these modified procedures have provided some technological advantages, *in vitro* produced embryos generally showed higher viability when frozen by vitrification than slow freezing, as demonstrated by a lower occurrence of morphological changes after thawing (Moreira da Silva and Metelo 2005). Our putative results coincide with the declaration of Massip et al. (1995) that, in addition to the morphology and developmental stage of embryos, the kinetics of embryo development and the age of embryos before freezing plays an important role in embryo survival after thawing. However, in contrast to these reports, we found out that the survival rate was dependent on the quality of embryos, not on the culture period of vitrification or freezing. Nevertheless the blastocyst hatching rate did significantly depend on the culture period or age of the embryos subjected to vitrification. The toxicity of CPA may be taken into account actually.

In this study, the G1 Exp-BLs at day 7 had a nearly similar rate of hatchability to the fresh embryos. In our study, we also investigated the correlationship between the post warming embryo survival rate and the hatching rate of embryos by curve estimation of correlation regression. We discovered that the hatching rates of G1 embryos at 48 h of the day 7 culture periods showed a significant and strong relationship to these embryos' development and post- warming survival rates.

However, our findings partially agreed with those of Vajta et al. (1995), Dinnyes et al. (1999) and Machatkova et al. (2006) that, in embryos of equal quality in terms of morphology, day 7 embryos showed higher cryosurvival rates. They, however, did not consider the grade of blastocysts or differences between culture periods in their studies, but did mention that the highest difference in cryosurvival rates was between day 7 and day 8 embryos at the early blastocyst stage. These differences disappeared when frozen-thawed embryos were at the blastocyst or expanded blastocyst stage. In our study we also found that the vitrified-thawed, lower quality (G2) expanded blastocysts after 48 h culture showed about 40% hatchability at day 7, which was significantly higher than that of day 8. Saha and Suzuki (1997) proposed that day 8 blastocysts had lower quality because of fewer inner cells. Results by Kong et al. (2000) and Mezzalira et al. (2004) suggest that day 8 embryos have important differences in their hatching rates.

The results of this study demonstrated that the survival rate of G1 Exp-BLs showed no difference between day 7 and day 8 culture periods but that day 7 embryos had a significantly higher hatchability rate subsequent to Cryotop vitrification. However, it was observed that rapidly growing embryos are more tolerant of the vitrification process than slower growing embryos. Therefore, we suggest that the day 7 Exp-BL is more suitable for cryopreservation and ET demonstrates the strongest co-relation between survival and hatching rates. In our next study, we will attempt to further confirm these findings by using additional tests involving ET.

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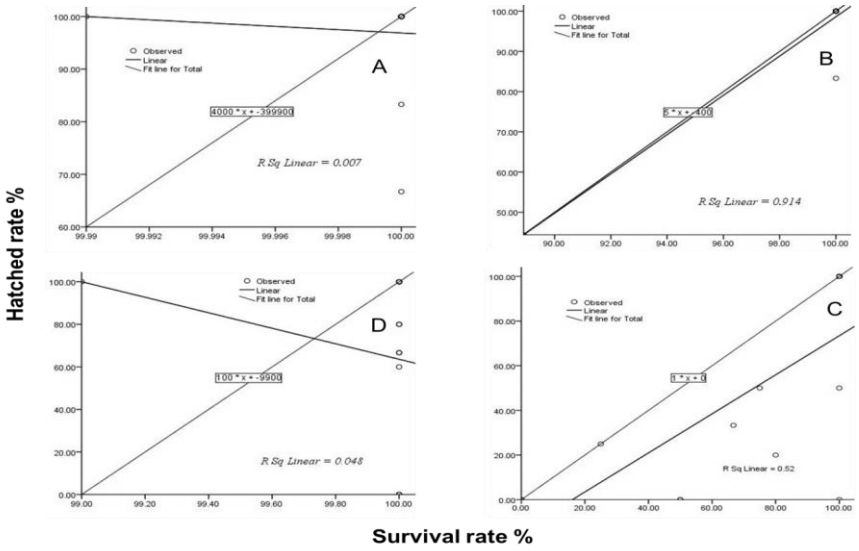

Figure 2 Curve estimation of correlation regressions between day 7 expanded blastocysts survival and hatching rates. X-axis survival rate and Y-axis hatching rate; (A): Fresh control day 7 G1 Exp-BLs, (B): Vitrified day 7 G1 Exp-BLs, (C): Fresh control day 7 G2 Exp-BLs and (D): Vitrified day 7 G2 Exp-BLs

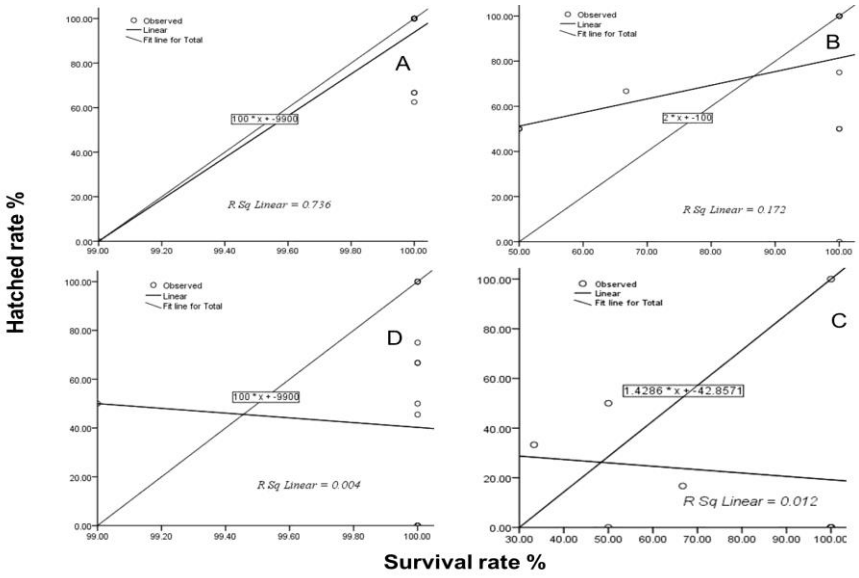

Figure 3 Curve estimation of correlation regressions between day 8 expanded blastocysts survival and hatching rates. X-axis survival rate and Y-axis hatching rate; (A): Fresh control day 8 G1 Exp-BLs, (B): Vitrified day 8 G1 Exp-BLs, (C): Fresh control day 8 G2 Exp-BLs and (D): Vitrified day 8 G2 Exp-BLs

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บทคัดย่อ

การศึกษาอัตราการเจริญของตัวอ่อนโคระยะ Expanded blastocyst หลังจากผ่านการแช่แข็งด้วยวิธี Cryotop vitrification

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การแช่แข็งตัวอ่อนเป็นส่วนหนึ่งของการวิจัยด้านระบบสืบพันธุ์ การศึกษาก่อนหน้านี้พบว่าความสามารถในการเจริญเติบโตของตัวอ่อนหลังจากแช่แข็งขึ้นกับระยะเวลาในการเลี้ยงตัวอ่อนก่อนที่จะทำการแช่แข็ง การทดลองนี้ศึกษาอัตราการรอดและการเจริญถึงระยะแฮชบลาสโตซีสของตัวอ่อนโคในหลอดแก้ว อายุ 7 และ 8 วัน ที่ผ่านการแช่แข็งด้วยวิธี Cryotop vitrification จากการศึกษาพบว่าอัตราการเจริญสู่ระยะบลาสโตซีสของตัวอ่อนอายุ 8 วัน (28.1%) สูงกว่าตัวอ่อนอายุ 7 วัน (19.1%) และเมื่อนำตัวอ่อนระยะบลาสโตซีสอายุ 7 และ 8 วัน เกรด 1 และเกรด 2 มาทำการแช่แข็งด้วยวิธี Cryotop โดยนำตัวอ่อนมาแช่ในน้ำยาแช่แข็งที่ประกอบด้วย 20% DMSO + 20% EG และ 0.5M Sucrose พบว่าอัตรารอดหลังการทำละลายของตัวอ่อนระยะบลาสโตซีสอายุ 7 วัน ทั้งเกรด 1 เกรด 2 และตัวอ่อนระยะบลาสโตซีสอายุ 8 วัน เกรด 1 ที่ทำการแช่แข็งด้วยวิธี Cryotop vitrification ไม่แตกต่างกันทางสถิติกับกลุ่มควบคุม ยกเว้นตัวอ่อนระยะบลาสโตซีสเกรด 2 อายุ 8 วันที่แตกต่างจากกลุ่มควบคุม และพบว่าอัตราการเจริญถึงระยะแฮชบลาสโตซีสหลังการทำละลายของตัวอ่อนระยะบลาสโตซีสที่แช่แข็งเมื่ออายุ 7 วันสูงกว่าที่แช่แข็งเมื่ออายุ 8 วัน จากการวิเคราะห์ทางสถิติด้วย correlation regression พบว่าอัตราการเจริญถึงระยะแฮชบลาสโตซีสหลังการทำละลายและเลี้ยงต่อเป็นเวลา 48 ชั่วโมงของตัวอ่อนระยะบลาสโตซีสแช่แข็งเกรด 1 อายุ 7 วันมีความสัมพันธ์กับอัตรารอดของตัวอ่อนหลังจากแช่แข็ง (R^2=0.914) จากการทดลองนี้สรุปได้ว่าตัวอ่อนระยะบลาสโตซีสที่ได้จากการปฏิสนธิในหลอดแก้วอายุ 7 วันเหมาะสมสำหรับการแช่แข็งด้วยวิธี vitrification มากกว่าตัวอ่อนอายุ 8 วัน

คำสำคัญ: การปฏิสนธิในหลอดแก้ว โค ตัวอ่อนระยะบลาสโตซีส การแช่แข็งแบบเนื้อแก้ว

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