Cryobiology 59 (2009) 285–290

Contents lists available at ScienceDirect

Cryobiology

journal homepage: www.elsevier.com/locate/ycryo

Effect of culture system on survival rate of vitrified bovine embryos produced in vitro ☆

A. Shirazi [a,b,*], H. Nazari [b,1], E. Ahmadi [b,1], B. Heidari [a,b], N. Shams-Esfandabadi [b,1]

[a] Department of Veterinary Biomedical Sciences, Western College of Veterinary Medicine, University of Saskatchewan, 52 Campus Drive, Saskatoon, Sask., Canada S7N 5B4
[b] Research Institute of Animal Embryo Technology, Shahrekord University, P.O. Box 115, Shahrekord, Iran

ARTICLE INFO

Article history:
Received 6 July 2009
Accepted 24 August 2009
Available online 28 August 2009

Keywords:
Vitrification
Bovine
Blastocyst
In vitro production
Co-culture

ABSTRACT

This study was designed to evaluate the effect of in vitro culture system on bovine blastocyst yield and quality after vitrification. In Experiment 1, IVM/IVF zygotes were allocated to three culture conditions: (I) Oviductal cells-SOF (OCM-SOF); (II) Oviductal cells-TCM (OCM-TCM); and (III) SOF for 8 days. There was no significant difference between blastocyst rates among groups.

In Experiment 2, the IVP-blastocysts in three above culture conditions were vitrified within groups segregated according to age (Day 7 and 8) and blastocoelic cavity size (early and expanded blastocysts). A trend of higher survival rate was obtained in vitrified/warmed early blastocysts compared with expanded ones, so that the difference in OCM-TCM group was significant ($P < 0.001$). Higher survival and hatching rates ($P < 0.001$) were obtained in OCM-SOF and OCM-TCM groups (co-culture) compared with SOF group and the age of blastocyst had no effect on post-thaw survival and hatching rates. In Experiment 3, after staining of blastocysts, in fresh blastocysts the highest number of trophectoderm cells was observed in OCM-TCM group and the number of inner cell mass (ICM) was higher in co-culture groups than SOF group ($P < 0.001$). In vitrified/warmed blastocysts the number of ICM and trophectoderm cells in co-culture groups was higher than SOF group ($P < 0.001$) except for the ICM of expanded blastocysts. In conclusion, in our culture conditions, the blastocyst yield is not influenced by culture system, while the cryotolerance of IVP-blastocysts is positively influenced by the presence of somatic cells. Moreover, the expanded blastocysts are more susceptible to cryoinjury than early blastocysts.

Introduction

The media, sera, and protocols used for in vitro production of bovine embryos are the main sources of variation in IVF programs. Irrespective of the origin and quality of the zygote, embryo development in vitro is influenced by a number of factors, such as co-culture with somatic cells [4], addition of anti-oxidants to the culture medium [1], oxygen tension [29], and the number of embryos present in the culture drop [10].

It has been shown that, while the intrinsic quality of the oocyte determines the proportion of oocytes developing to blastocysts, it is the post-fertilization culture environment that has the biggest influence on blastocyst quality especially on the timing of development, hatchability, and total cell numbers [15,21]. All of these factors might contribute considerably to their greater sensitivity to cryoinjury, and to the reduced pregnancy rates following embryo transfer [9]. In addition, it is known that the conditions of culture in vitro can alter gene expression in the embryo [14,17].

Clearly, any modifications of the culture environment, which could affect any or all of these processes, could have a major effect on the quality of the embryo and its sensitivity to cryoinjury. In this context, it has been shown that by culturing in vitro produced bovine zygotes in ewe oviduct, it is possible to increase markedly the quality of the resulting blastocysts, measured in terms of cryotolerance, to a level similar to that of embryos produced entirely in vivo [6,21]. As a result, any detrimental effects can be seen as blastocysts incapable of withstanding cryopreservation.

Simplification of in vitro culture conditions offers the advantage of limiting problems due to variation in composition of biological fluids and co-culture with epithelial cells. There are also evidence indicating embryo culture in synthetic oviductal fluid (SOF) has resulted in a significantly higher blastocyst yield on both Day 7 and 8 than did culture in TCM199-GCM (TCM199-granulosa cells mono layer) and SOF-GCM. Although, after vitrification significantly more blastocysts produced in TCM199-GCM or SOF-GCM survived at 72 h of culture compared to those produced in SOF [22].

☆ This study has been financially supported by the Research Institute of Animal Embryo Technology, Shahrekord University, Shahrekord, Iran.

* Corresponding author. Address: Department of Veterinary Biomedical Sciences, Western College of Veterinary Medicine, University of Saskatchewan, 52 Campus Drive, Saskatoon, Sask., Canada S7N 5B4. Fax: +1 306 966 7376.
E-mail addresses: abs890@mail.usask.ca, shiraziabbas@yahoo.com (A. Shirazi).
[1] Fax: +98 381 4424412.

0011-2240/$ - see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.cryobiol.2009.08.003

From the above, it is clear that any improvement in the quality of blastocysts produced in vitro is likely to derive from the modification of the post-fertilization culture conditions. Thus, it would be important to compare the efficacy of various successfully used in vitro culture protocols for embryo competence and viability after vitrification. Since the speed of early embryo development is related to the quality of the embryo, in the current study beside the evaluation of the effect of culture system on sensitivity of in vitro produced (IVP) bovine embryos to cryoinjury, the effects of age of blastocyst and its expansion on freezability of IVP-embryos are assessed.

Materials and methods

Except where otherwise indicated, all chemicals were obtained from the Sigma (St. Louis, MO, USA).

In vitro embryo production

The ovaries were collected at a local slaughterhouse and transported to the laboratory within 2–3 h in normal saline at temperature between 30 and 35 °C. Ovaries were washed three times with prewarmed fresh saline (37 °C), and all visible follicles with a diameter of 2–8 mm were aspirated using gentle vacuum (30 mm Hg) via a 18 gauge short beveled needle connected to a 10 ml syringe. The follicle content was released in preincubated hepes-TCM, supplemented with penicillin and streptomycin and 50 IU/ml heparin.

The cumulus–oocyte complexes (COCs) with at least three layers of cumulus cells, oocytes with a uniform granulated cytoplasm, homogenous distribution of lipid droplets in the cytoplasm were selected for the experiments. The selected COCs were in vitro matured in TCM199 supplemented with 10% FBS (Fetal bovine serum, Gibco 10270), 0.02 mg/ml cysteamine and 0.1 IU/ml FSH. Ten to 15 COCs were transferred in 50 μl of the maturation medium in a 60 mm Petri dish (Falcon 3004; Becton & Dickinson, Franklin Lakes, NJ), layered with sterile mineral oil and cultured for 24 h in 5% CO_2 in air at 39 °C. The matured oocytes were exposed to motile spermatozoa obtained by centrifugation of frozen–thawed semen on a discontinuous Percoll density gradient (1 ml 40% Percoll over 1 ml 90% Percoll) at 700*g* for 20 min. Oocytes were cultured in TALP medium supplemented with 6 mg/ml BSA and incubated with motile spermatozoa at 1×10^6 spermatozoa/ml concentration layered with mineral oil, for 22–24 h at 39 °C in an atmosphere of 5% CO_2 in air. After fertilization, presumptive zygotes were mechanically denuded of their cumulus cells and cultured in three different culture systems: (I) Oviductal cells-monolayer (OCM)-synthetic oviductal fluid supplemented with amino acid and bovine serum albumin (SOFaaBSA) (OCM-SOF) under mineral oil in 5% CO_2 in maximum humidified atmosphere; (II) Oviductal cells-monolayer-TCM199 (OCM-TCM) under mineral oil in 5% CO_2 in maximum humidified atmosphere; and (III) SOFaaBSA (SOF) in 5% CO_2, 7% O2, 88% N2 for 9 days.

Vitrification and warming procedures

Al1 vitrification solutions were prepared using PBS, antibiotics, 0.3 mM sodium pyruvate, 3.3 mM glucose, and 20% (v/v) FCS (Sigma). The IVP-blastocysts were sequentially exposed to corresponding equilibration and vitrification solutions at room temperature (25 °C). For equilibration, the embryos ($n = 5$) were placed into the first 100 μl drop of equilibration solution containing glycerol 1.4 M for 7 min, and then transferred into the second 100 μl drop of equilibration solution (glycerol 1.4 M and ethylene glycol 3.6 M) for 3 min. The embryos were then transferred into a column of vitrification solution (3.4 M glycerol and 4.6 M ethylene glycol) at the center of 0.25 ml straws using a fine glass capillary pipette. The column of vitrification solution in the straws was separated by two air bubbles from two columns of 1 M sucrose solution. The straws were sealed and then plunged immediately into LN2 and maintained until use. The time limit for the exposure of embryos to the vitrification solution and the immersion of straws into LN2 was 45 s. For evaluation of post vitrification embryo survival rate the straws were warmed by being transferred from LN2 to the air for 10 s and then immersed into a water bath at 25–30 °C for 8 s. The contents of each straw were then expelled into warming solution containing 1 M sucrose, the medium was stirred gently to facilitate mixing of the two solutions. The manipulated blastocysts were then transferred into 100 μl drops of sucrose solution (0.5 M) for 5 min to allow the removal of intracellular cryoprotectants, and then washed in PBS. The vitrified/warmed blastocysts were cultured in OCM-SOF in 5% CO_2 until hatched blastocyst stage. Post warming viability of blastocysts was assessed by their re-expansion, resumption of cellular division and hatching.

Staining

Blastocysts were incubated for 15 min at 39 °C in the base medium (Hepes-synthetic oviductal fluid; H-SOF containing 5 mg/ml BSA) supplemented with 10 mg/ml propidium iodide (PI). They were then transferred to a drop of base medium on a glass slide and examined under an epifluorescent microscope (IX71 Olympus, Tokyo, Japan). The trophectoderm (TE) cells with membrane lesion (dead cells) were stained with PI. Indeed, PI only enters cells with altered membrane integrity (red color following UV excitation).

For differential staining of ICM and TE cells compartments the blastocysts which had been stained with PI were incubated in Triton X-100 prepared in the base medium for 20 s. The blastocysts were then stained in the base medium containing 30 μg/ml PI for 1 min. After two washes in the base medium, the blastocysts were transferred in ice-cold ethanol containing 10 μg/ml Hoechst 33342 for 15 min. The blastocysts were directly mounted into the small droplet of glycerol on glass slide and examined under an epifluorescent microscope.

For cell counting, the flattened blastocysts under coverslip compression were visualized sequentially with moderate and high magnification of an epifluorescent microscope. Moreover, the photograph of each blastocyst was then inspected and the number of ICM and TE cells was determined. ICM nuclei appeared blue, caused by DNA labeling with the membrane permeable Hoechst 33342, and TE cells appeared red due to staining of nuclear DNA with the membrane impermeable PI.

Experimental design

Experiment 1: In vitro production of bovine blastocyst in different culture systems

A total of 4718 IVM/IVF oocytes were used in at least eighteen replicates. After fertilization, the presumptive zygotes were randomly allocated (6 embryos/30 μl drop) into three different culture systems as follows: (I) OCM-SOF; (II) OCM-TCM; and (III) SOF. For all treatments embryo morphology was evaluated on Days 2, 5, 7, 8 and 9 to determine stage of development.

Experiment 2: Survival and hatchability rate of embryos cultured in different culture systems after vitrification

The quality of blastocysts produced in three different culture systems considering the blastocoelic cavity size (early and expanded blasocysts) and age of early and expanded blastocysts (Day 7 vs. Day 8) were evaluated. The in vitro produced blastocysts based on blastocoelic cavity size and their age were randomly allo-

cated into each experimental group and then vitrified. One week after vitrification, the embryos were warmed and cultured in OCM-SOF. Twenty four and 72 h after thawing the embryos were morphologically evaluated, for assessment of survival and hatching rates.

Experiment 3: Evaluation of ICM, TE, and dead cells of blastocysts derived from embryos cultured in different culture systems before and after vitrification

This experiment was design to evaluate the cumulative effect of culture system, size of blastocoelic cavity, and age of blastocyst on cryotolerance of IVP-blastocysts in term of cellular compartments. The culture systems used and the number of experimental groups were the same as those described for Experiment 2. In each experimental group the cellular compartments of fresh blastocysts and vitrified/warmed blastocysts after 48–72 h of culture were differentially stained.

Statistical analysis

Data was collected over at least five replicates. All proportional data (cleavage and blastocyst formation rates) were subjected to an arc-sine transformation, and the transformed values were analyzed using one-way ANOVA. The blastocyst cell numbers in different experimental groups were analyzed using ANOVA. When ANOVA revealed a significant effect, comparison of means among groups was performed using Tukey test. The Kolmogorov–Smirnov test (with Lilliefors' correction) was applied to test data for normality. When normality test was failed the Kruskal–Wallis One Way Analysis of Variance on Ranks was applied. To isolate the group(s) that was differed from the others, All Pairwise Multiple Comparison Procedures (Dunn's Method) was applied. Comparisons of vitrified-warmed blastocyst survival and hatching rates between groups were carried out by the Chi-squared test. A $P < 0.05$ level was considered significant (Jandel SigmaStat soft ware, Version 2.0, 1995). Data was expressed as mean ± SEM.

Results

Embryo development

The proportion of oocytes that cleaved 48 h post-fertilization in oocytes cultured in SOF was higher than those cultured in OCM-TCM. There was, however, no effect of culture media on the overall blastocyst yield at Day 9 (range 28.5–31.1%; Table 1) among three groups.

Survival of vitrified/warmed IVP-embryos

The highest and lowest survival rates of vitrified/warmed blastocysts (24 h after warming) were observed in embryos that had been developed and reached blastocyst in OCM-TCM and SOF, respectively (Table 2). In all culture conditions there was a trend of a lower survival and hatching rates in expanded blastocysts compared with blastocysts. There was, however, no difference in embryo survival and hatching rates between Day 7 and 8 of both blastocysts and expanded blastocysts. The survival of vitrified/warmed early and expanded blastocysts of those embryos developed in SOF as well as their hatching rates were the lowest among all experimental groups (Table 2).

Table 1
The effect of different culture systems on development of IVP bovine embryos.

Culture conditions	Replicates	Cultured oocytes	Cleavage	Blastocyst
	n	*n*	*n* (mean ± SEM)	
OCM-SOF	18	1222	898 (74.6 ± 0.01)[a,b]	379 (31.1 ± 0.01)
OCM-TCM	23	1429	1038 (71.4 ± 0.01)[a]	416 (28.5 ± 0.01)
SOF	38	2067	1551 (75.3 ± 0.01)[b]	656 (28.9 ± 0.01)

[a,b] Numbers with different superscripts in the same column differ significantly ($P < 0.05$).

Blastocyst cell allocation to trophectoderm and inner cell mass

The numbers of TE cells and ICM of both fresh and vitrified/warmed early and expanded blastocysts were affected by culturing of zygotes in different culture conditions (Table 3).

Fresh embryos that developed and reached early and expanded blastocyst stages in OCM-TCM had the greatest ($P < 0.001$) number of TE cells compared with those cultured in OCM-SOF and SOF. The ICM number of both blastocysts and expanded blastocysts developed in OCM-SOF and OCM-TCM was greater than those developed in SOF. In vitrified/warmed embryos, except for the ICM of expanded blastocysts, the numbers of TE cells and ICM were greater for those early blastocysts and expanded blastocysts developed in OCM-SOF and OCM-TCM than SOF (Table 3).

There was a trend of an increased number of TE cells in vitrified/warmed Day 7 and 8 early blastocysts over 72 h culture compared with corresponding values in fresh blastocysts for all culture conditions except for Day 8 early blastocysts in SOF. In contrast, the numbers of TE cells in vitrified/warmed Day 7 and 8 expanded blastocysts developed in OCM-TCM and SOF was significantly lower than fresh counterparts. The difference, however, was insignificant for those expanded blastocysts developed in OCM-SOF. In all culture conditions and for both vitrified/warmed early and expanded blastocysts the numbers of ICM were lower than corresponding values in fresh counterparts. The difference was more obvious for Day 8 expanded blastocysts (Table 4).

The proportion of dead cells

The proportions of dead cells in vitrified/warmed early and expanded blastocysts developed in different culture conditions, considering the age of embryos (Day 7 or Day 8), were significantly higher than corresponding values in fresh counterparts. The difference, however, for the Day 8 early blastocyst developed on OCM-SOF was insignificant. The greatest proportion of dead cells in fresh and vitrified/warmed embryos was observed in Day 8 early blastocysts developed on OCM-SOF and SOF, respectively (Table 5).

Discussion

Within in vitro systems, modifications of the embryo culture environment after fertilization can have profound effects on gene expression in the embryo [18,20,31] which, in turn, may have serious implications for the normality of the blastocyst and their cryotolerance. In the current study the proportion of oocytes that cleaved 48 h post-fertilization was influenced by the culture condition, so that the rate of cleavage was higher in oocytes cultured in SOF compared with OCM-TCM. The blastocyst rate, however, was not affected by culture conditions.

In this context, the culture of bovine embryos in SOF medium resulted in a significantly higher blastocyst yield on both Day 7 ($P < 0.001$) and 8 ($P < 0.001$) than did culture in TCM199-GCM (granulose cell monolayer) and SOF-GCM [22].

As known, co-culture with somatic cells improves embryo development not only through secretion of growth factors but also

Table 2
The effect of different culture systems, blastocoelic cavity size, and age of blastocyst on survival rate of vitrified/warmed IVP bovine blastocysts.

Culture conditions	Embryonic stage	Age of blastocyst (days)	No. of vitrified embryos	Survived embryos *n* (%)	Hatched embryos *n* (%)
OCM-SOF	Early blast.	7	68	52 (76.5)[a,b,c]	38 (73.1)[a]
		8	72	55 (76.6)[a,b,c]	39 (70.9)[a]
	Exp. blast.	7	73	51 (69.9)[a,b,c]	31 (60.8)[a]
		8	63	40 (63.5)[a,c]	23 (57.5)[a]
OCM-TCM	Early blast.	7	69	57 (82.6)[b]	41 (71.9)[a]
		8	65	52 (80.0)[b,a]	36 (69.2)[a]
	Exp. blast.	7	58	36 (62.1)[c]	22 (61.1)[a]
		8	67	41 (61.2)[c]	25 (61.0)[a]
SOF	Early blast.	7	82	25 (30.5)[d]	5 (20.0)[b]
		8	57	16 (28.1)[d]	3 (18.8)[b]
	Exp. blast.	7	63	14 (22.2)[d]	1 (17.1)[b]
		8	49	10 (20.4)[d]	0 (0.0)[b]

[a,b,c,d] Numbers with different superscripts in the same column differ significantly ($P < 0.001$).

Table 3
The effect of different culture systems and blastocoelic cavity size on cell numbers (mean ± SEM) of fresh and vitrified/warmed IVP bovine blastocysts.

Culture conditions	Fresh				Vitrified-warmed			
	Early blast.		Exp. blast.		Early blast.		Exp. blast.	
	TE	ICM	TE	ICM	TE	ICM	TE	ICM
OCM-SOF	32.4 ± 2.8[a]	27.2 ± 2.7[a]	104.3 ± 9[a]	33.6 ± 3.1[a]	63.5 ± 10.4[a]	17.5 ± 3.2[a]	86.7 ± 9.1[a]	18.9 ± 2.7
OCM-TCM	49.3 ± 3.6[b]	26.3 ± 2.7[a]	130.5 ± 6.6[b]	35.7 ± 2.7[a]	54.4 ± 5.2[a]	20.4 ± 3.5[a]	82.6 ± 9[a]	21.4 ± 3.5
SOF	31.4 ± 3[a]	11.8 ± 1.2[b]	83.5 ± 5.3[c]	20.2 ± 1.9[b]	26.6 ± 3[b]	6.9 ± 1.8[b]	39.1 ± 3.2[b]	12.3 ± 1.7

[a,b,c] Numbers with different superscripts in the same column differ significantly ($P < 0.001$).

Table 4
The effect of different culture systems, blastocoelic cavity size (early vs. expanded blastocyst), and blastocyst age on cell numbers (mean ± SEM) of fresh and vitrified/warmed IVP bovine blastocysts.

Culture conditions	Embryonic stage	Age of blastocyst (days)	Trophectoderm cell		Inner cell mass	
			Fresh	Vitrified-warmed	Fresh	Vitrified-warmed
OCM-SOF	Early blast.	7	27.7 ± 1.8[a]	74.4 ± 18[b]	27.2 ± 4.2	18.4 ± 4.7
		8	38.4 ± 5.3	52.6 ± 10	27.1 ± 3.3	16.6 ± 4.9
	Exp. blast.	7	89.6 ± 8.4	81.6 ± 8.5	32.9 ± 3.9[A]	20.2 ± 4.2[B]
		8	125.4 ± 16.5	92.2 ± 17.1	34.7 ± 5.2[A]	17.4 ± 3.5[B]
OCM-TCM	Early blast.	7	41.4 ± 4.4	53.1 ± 7.6	26.2 ± 2.9	23.8 ± 4.4
		8	58.4 ± 3.6	56.8 ± 5.7	26.3 ± 5.1	14.2 ± 5.5
	Exp. blast.	7	112.8 ± 6.3[a]	65.8 ± 6.3[b]	33.9 ± 3	24.8 ± 5.3
		8	149.7 ± 9.2[a]	107 ± 17.5[b]	37.6 ± 4.7[A]	16.3 ± 3.4[B]
SOF	Early blast.	7	24.4 ± 2.7	27.8 ± 4.7	13 ± 1.5	7 ± 2.9
		8	39.5 ± 3.6[a]	24.7 ± 3.2[b]	10.5 ± 1.7	6.7 ± 1.7
	Exp. blast.	7	62.5 ± 5.8[a]	44.9 ± 4.2[b]	17.1 ± 2.5	15.1 ± 2.3
		8	96.1 ± 6.5[a]	30.4 ± 3.3[b]	22 ± 2.7[A]	8.1 ± 1.6[B]

[a,b] Numbers with different lowercase letters in the same row differ significantly ($P < 0.05$).
[A,B] Numbers with different uppercase letters in the same row differ significantly ($P < 0.05$).

through glucose reduction caused by their metabolism [8,19]. Additionally, in bovine the embryo block at the 8- to 16-cell stage can be overcome by culturing embryos with various somatic cells such as bovine cumulus/granulosa or oviductal cells [5,7,24]. It has also been reported that bovine co-culture derived blastocysts at 72 h post vitrification have higher survival compared to those developed and reached to blastocyst in SOF [22].

In the current study, the lower survival rate of vitrified/warmed expanded blastocysts compared with early blastocysts in all three culture conditions confirmed the results obtained in human blastocysts indicating a decrease in survival rate of vitrified/warmed blastocysts when the volume of the blastocoelic cavity was increased [28]. In human, the efficiency of the vitrification method was dependent on the stage of embryo development and was negatively correlated with the expansion of the blastocoele. It seems in expanded blastocysts because of the large blastocoele and the short exposure time to the cryoprotectant solution, a low concentration of permeable cryoprotectant is present inside the cavity, probably not sufficient to protect the blastocysts against formation of ice crystals inside the blastocoele [28]. This conclusion, however, does not support what has been suggested by Vajta et al. [27] indicating the easy permeation of cryoprotectant agents to both TE and ICM of expanded or hatching blastocyst. Moreover, whereas in the current study the vitrification procedure was carried out in stepwise manner (2 steps in equilibration solutions and 1 step in vitrification solution), it seems the exposure time to the cryoprotectant

Table 5
The effect of different culture systems, blastocoelic cavity size, and age of blastocyst on proportion of dead cells in fresh and vitrified/warmed IVP bovine blastocysts.

Culture conditions	Embryonic stage	Blastocyst age (days)	Embryonic cells (mean ± SEM)					
			Fresh			Vitrified-warmed		
			Dead	Total	Dead/total*	Dead	Total	Dead/total*
OCM-SOF	Early blast.	7	4 ± 0.7	52.8 ± 3.9	$^{A,B}0.09^{a}$ (0.05–0.1)	15.5 ± 1.7	91.5 ± 8.2	$^{A}0.14^{b}$ (0.12–0.26)
		8	6.6 ± 1.6	51.4 ± 5.8	$^{B}0.14$ (0.06–0.2)	17.3 ± 2.5	82.8 ± 10.1	$^{A}0.2$ (0.19–0.24)
	Exp. blast.	7	5 ± 0.9	109.9 ± 13.7	$^{A}0.05^{a}$ (0.04–0.07)	15.5 ± 2.2	101.6 ± 8.2	$^{A}0.2^{b}$ (0.1–0.23)
		8	6.7 ± 1.3	118 ± 11.1	$^{A}0.05^{a}$ (0.03–0.08)	19.9 ± 2.9	110.7 ± 9.4	$^{A}0.19^{b}$ (0.12–0.21)
OCM-TCM	Early blast.	7	4.4 ± 0.7	64.8 ± 4.1	$^{A,B}0.06^{a}$ (0.05–0.08)	15.7 ± 1.6	65.5 ± 6	$^{A,B}0.24^{b}$ (0.18–0.33)
		8	6.8 ± 0.9	$^{A,B}0.1^{a}$ (0.09–0.1)	70.3 ± 3.6	17.5 ± 1.8	68.8 ± 6.6	$^{A,B}0.24^{b}$ (0.21–0.3)
	Exp. blast.	7	5.7 ± 1.3	128.3 ± 7.8	$^{A}0.05^{a}$ (0.02–0.07)	16.5 ± 2.9	93 ± 8.8	$^{A}0.18^{b}$ (0.11–0.23)
		8	8.3 ± 1.2	131.5 ± 10.3	$^{A}0.06^{a}$ (0.05–0.08)	19 ± 2.2	105.7 ± 9.2	$^{A}0.17^{b}$ (0.16–0.2)
SOF	Early blast.	7	2.9 ± 0.6	55.4 ± 4	$^{A}0.05^{a}$ (0.03–0.08)	14 ± 2.3	36.3 ± 3.7	$^{A,B}0.41^{b}$ (0.2–0.6)
		8	4.1 ± 0.9	52.1 ± 3.9	$^{A,B}0.08^{a}$ (0.04–0.1)	15.7 ± 2.1	33.7 ± 3.9	$^{B}0.45^{b}$ (0.32–0.67)
	Exp. blast.	7	4.2 ± 0.6	93.4 ± 6.2	$^{A}0.05^{a}$ (0.04–0.05)	18.3 ± 2.5	55.8 ± 9.6	$^{A,B}0.3^{b}$ (0.24–0.46)
		8	5.5 ± 1	97.7 ± 5.4	$^{A}0.06^{a}$ (0.03–0.06)	15.5 ± 2	39.3 ± 3.7	$^{A,B}0.45^{b}$ (0.28–0.52)

^{a,b} Means ± SEM; different lowercase letters indicate statistical differences within rows ($P < 0.05$).
^{A,B} Means ± SEM; different uppercase letters indicate statistical differences within columns ($P < 0.05$).
* Values represent median change expressed as percentage, with interquartile range (25th–75th percentile) in parentheses.

solution has been sufficient to allow adequate concentration of permeable cryoprotectant inside the blastocoele. It has also been shown that microsuction of blastocoelic fluid before vitrification increased the survival rates for mouse early (92% vs. 80%) and expanded blastocysts (89% vs. 59%) [3]. In contrast, the vitrified/warmed ovine early blastocysts were less viable than expanded ($P < 0.01$), hatching or hatched blastocysts [16]. Meanwhile, the viability of ovine and bovine embryos at the expanded blastocysts stage has been found to be higher than at the morula stage because the embryonic tolerance to cooling increases after formation of the blastocoele [27]. Despite the above controversy, in the current study the expanded blastocysts were more sensitive to cryoinjury induced by vitrification/thawing procedure compared to the early blastocysts (Table 2).

Despite our general knowledge indicating that at the same embryonic age, a larger size implies a more advanced degree of embryonic development, which could positively affect the efficiency of the vitrification procedure [12,28], in the current study there was no such a correlation among Day 7 and 8 of early and expanded blastocysts. Instead, at the same embryonic age the cryotolerance of more advanced embryos (expanded blastocysts), at least in some groups, was less than embryos at the early blastocyst stage.

In the current study the quality of in vitro produced early and expanded blastocysts, assessed by their cryotolerance, hatchability rate and the number of ICM and TE cells, was affected by culture conditions. As shown, the lowest rates of survival and hatchability in vitrified/warmed early and expanded blastocysts was observed in SOF derived embryos among three different culture conditions (Tables 2–4). Actually, the expanded blastocysts developed in SOF and early blastocysts produced in OCM-TCM had the lowest and highest rates of survival over 72 h after warming, respectively (Table 2). The decrease in hatching rate might be related to the lower cell numbers in SOF derived blastocysts by comparison with those derived from co-culture system (Tables 2 and 3). Likewise, the numbers of ICM and TE cells in fresh and vitrified/warmed early and expanded blastocysts were lowest in SOF group except for TE cells in fresh early blastocysts and ICM in vitrified/warmed expanded blastocysts, compared with OCM-SOF and OCM-TCM groups (Table 3).

The numbers of ICM and TE cells in vitrified/warmed early blastocysts in OCM-TCM groups were comparable with the corresponding numbers in vitrified/warmed bovine blastocysts (20.4 ± 3.5 and 54.4 ± 5.2 vs. 22 ± 1 and 56 ± 6) in another study [13].

Embryos that developed and reached to the blastocyst stage in OCM-TCM had the highest (<0.001) TE numbers in both early and expanded blastocysts compared with those cultured in either OCM-SOF or SOF medium. In early blastocysts, following vitrification/warming procedures the numbers of TE cells over 72 h after warming were either unchanged or increased except for Day 8 early blastocysts which had been developed in SOF. In contrast, the numbers of TE cells in expanded blastocysts were decreased by vitrification/warming procedures, regardless of their respective culture conditions. Indeed, despite re-expansion of vitrified/warmed expanded blastocysts, the TE cells could not properly proliferate by comparison with vitrified/warmed early blastocysts (Tables 3 and 4). The decrease in the number of TE cells in expanded blastocysts and ICM in both early and expanded blastocysts after cryopreservation was in agreement with other reports (2, 11). In vitrified/warmed in vivo derived mouse blastocysts, however, the numbers of ICM and TE cells have not been influenced by the cryopreservation procedure [23]. In feline, the cryopreservation procedure had no negative effect on the number of ICM and TE cells in vitrified blastocysts produced in vitro [26].

In the current study, the marked decrease in TE cell numbers in expanded blastocysts could be correlated to their higher sensitivity to necrosis and apoptosis induced by vitrification/warming procedure compared to the early blastocysts as demonstrated by Baguisi et al. [2]. This decrease, however, could also be due to a slowing in embryonic cell proliferation after cryopreservation particularly in expanded blastocysts [25].

The numbers of ICM in both vitrified/warmed early and expanded blastocysts were decreased in all culture conditions, probably, due to the higher sensitivity of ICM to cellular degeneration compared to the TE cells in early blastocysts, during the cryopreservation procedure. The decrease was more prominent in expanded blastocysts which once more showed the greater sensitivity of expanded blastocysts to cryoinjury compared with early blastocysts.

In the current study the higher sensitivity of ICM to cryopreservation than TE cells in early blastocysts was contrary to the report [11] indicating the greater sensitivity of TE cells to vitrification compared with ICM. Whether at early blastocyst stage there is a difference in plasma membrane Na/K ATPase activity in favor of TE cells compared with ICM and, if so, the extent this difference

could affect the active transport mechanisms of cryoprotectants across cellular membrane, should be further investigated. As documented the increase in Na/K ATPase activity which occurs during blastocoelic formation in trophoblastic cells [30] may determine more active transport mechanisms of cryoprotectants leading to decreased exposure time and low concentration of cryoprotectants needed during freezing and thawing procedures [30].

The proportion of dead cells was negatively ($P < 0.05$) influenced by cryopreservation procedure. As expected, not all of the individual cells of blastocysts survive the cryopreservation process.

The greatest proportion of dead cells was observed in vitrified/warmed early blastocysts in SOF group which once more confirmed the inferior quality of blastocysts produced in this culture medium (Table 5).

In conclusion, the culture conditions have profound effect on the quality of in vitro produced bovine embryos in terms of their cryotolerance, hatchability rate, and blastocyst cell numbers. In co-culture system, the quality of IVP-embryos is positively influenced by the presence of somatic cells. Moreover, the expanded blastocysts are more sensitive than early blastocysts to cryoinjury induced by vitrification/warming procedure.

Acknowledgments

The authors thank the Research Institute of Animal Embryo Technology for technical and financial supports, Shahrekord University, and Shahrekord's slaughterhouse staff for their cooperation.

References

[1] A.A. Ali, J.F. Bilodeau, M.A. Sirard, Antioxidant requirements for bovine oocytes varies during in vitro maturation, fertilization and development, Theriogenology 59 (2003) 939–949.
[2] A. Baguisi, P. Lonergan, E.W. Overstrom, M.P. Boland, Vitrification of bovine embryos: incidence of necrosis and apoptosis, Theriogenology 51 (1999) 162.
[3] S.U. Chen, H.H. Lee, Y.R. Lien, Y.Y. Tsai, L.J. Chang, Y.S. Yang, Microsuction of blastocoelic fluid before vitrification increased survival and pregnancy of mouse expanded blastocysts, but pretreatment with the cytoskeletal stabilizer did not increase blastocyst survival, Fertil. Steril. 84 (2005) 1156–1162.
[4] I. Donnay, A. Van Langendonckt, P. Auquier, B. Grisart, A. Vansteenbrugge, A. Massip, F. Dessy, Effects of co-culture and embryo number on the in vitro development of bovine embryos, Theriogenology 47 (1997) 1549–1561.
[5] J.E. Ellington, E.W. Carney, P.B. Farrell, M.E. Simkin, R.H. Foote, Bovine 1–2-cell embryo development using a simple medium in three oviduct epithelial cell coculture systems, Biol. Reprod. 43 (1990) 97–104.
[6] B.P. Enright, P. Lonergan, A. Dinnyes, T. Fair, F.A. Ward, X. Yang, M.P. Boland, Culture of in vitro produced bovine zygotes in vitro versus in vivo: implications for early embryo development and quality, Theriogenology 54 (2000) 659–673.
[7] W.H. Eyestone, N.L. First, Co-culture of early cattle embryos to the blastocyst stage with oviductal tissue or in conditioned medium, J. Reprod. Fertil. 85 (1989) 715–720.
[8] Y. Fukuda, Development of early bovine embryos by co-culture: development to the blastocyst stage by co-culture with cumulus cells, J. Reprod. Dev. 38 (1992) 157–164.
[9] J.F. Hasler, In-vitro production of cattle embryos: problems with pregnancies and parturition, Hum. Reprod. 15 (2000) 47–58.
[10] P.J.M. Hendriksen, M.M. Bevers, S.J. Dieleman, Single IVP using BRL cell co-culture and serum yields a lower blastocyst rate than group culture, Theriogenology 51 (1999) 319.
[11] S. Kaidi, S. Bernard, P. Lambert, A. Massip, F. Dessy, I. Donnay, Effect of conventional controlled-rate freezing and vitrification on morphology and metabolism of bovine blastocysts produced in vitro, Biol. Reprod. 65 (2001) 1127–1134.
[12] S. Kaidi, I. Donnay, P. Lambert, F. Dessy, A. Massip, Osmotic behaviour of in vitro produced bovine blastocysts in cryoprotectant solutions as a potential predictive test of survival, Cryobiology 41 (2000) 106–115.
[13] S. Kaidi, I. Donnay, A. Van Langendonckt, F. Dessy, A. Massip, Comparison of two co-culture systems to assess the survival of in vitro produced bovine blastocysts after vitrification, Anim. Reprod. Sci. 52 (1998) 39–50.
[14] K.F. Lee, J.F.C. Chow, J.S. Xu, S.T.H. Chan, S.M. Ip, W.S.B. Yeung, A comparative study of gene expression in murine embryos developed in vivo, cultured in vitro, and cocultured with human oviductal cells using messenger ribonucleic acid differential display, Biol. Reprod. 64 (2001) 910–917.
[15] P. Lonergan, H. Khatir, F. Piumi, D. Rieger, P. Humblot, M.P. Boland, Effect of time interval from insemination to first cleavage on the developmental characteristics, sex ratio and pregnancy rate after transfer of bovine embryos, J. Reprod. Fertil. 117 (1999) 159–167.
[16] S. Naitana, S. Ledda, P. Loi, G. Leoni, L. Bogliolo, M. Dattena, P. Cappai, Polyvinyl alcohol as a defined substitute for serum in vitrification and warming solutions to cryopreserve ovine embryos at different stages of development, Anim. Reprod. Sci. 48 (1997) 247–256.
[17] D.R. Natale, P.A. De Sousa, M.E. Westhusin, A.J. Watson, Sensitivity of bovine blastocyst gene expression patterns to culture environments assessed by differential display RT-PCR, Reproduction 122 (2001) 687–693.
[18] S. Rief, F. Sinowatz, M. Stojkovic, R. Einspanier, E. Wolf, K. Prelle, Effects of a novel co-culture system on development, metabolism and gene expression of bovine embryos produced in vitro, Reproduction 124 (2002) 543–556.
[19] D. Rieger, M. Loskutoff, K.J. Betteridge, Developmentally related changes in the metabolism of glucose and glutamine by cattle embryos produced and co-cultured in vitro, J. Reprod. Fertil. 95 (1992) 585–595.
[20] D. Rizos, A. Gutierrez-Adan, S. Perez-Garnalo, J. de la Fuente, M.P. Boland, P. Lonergan, Bovine embryo culture in the presence or absence of serum: implications for blastocyst development, cryotolerance, and messenger RNA expression, Biol. Reprod. 68 (2003) 236–243.
[21] D. Rizos, P. Lonergan, F. Ward, P. Duffy, M.P. Boland, Consequences of bovine oocyte maturation, fertilization or early embryo development in vitro versus in vivo: implications for blastocyst yield and blastocyst quality, Mol. Reprod. Dev. 61 (2002) 234–248.
[22] D. Rizos, F. Ward, M.P. Boland, P. Lonergan, Effect of culture system on the yield and quality of bovine blastocysts as assessed by survival after vitrification, Theriogenology 56 (2001) 1–16.
[23] C.B. Sheehan, M. Lane, D.K. Gardner, The CryoLoop facilitates re-vitrification of embryos at four successive stages of development without impairing embryo growth, Hum. Reprod. 21 (2006) 2978–2984.
[24] M.A. Sirard, S. Bilodeau, Effects of granulosa cell co-culture on in-vitro meiotic resumption of bovine oocytes, J. Reprod. Fertil. 89 (1990) 459–465.
[25] M. Takagi, I. Sakonju, T. Suzuki, Effects of cryopreservation on DNA synthesis in the inner cell mass of in vitro matured/in vitro fertilized bovine embryos frozen in various cryoprotectants, J. Vet. Med. Sci. 58 (1996) 1237–1238.
[26] T. Tsujioka, C. Otzdorff, J. Braun, S. Hochi, Effect of post-IVF developmental kinetics on in vitro survival of vitrified-warmed domestic cat blastocysts, Reprod. Domest. Anim. 43 (2008) 323–327.
[27] G. Vajta, P. Holm, T. Greve, H. Callesen, Factors affecting survival rates of in vitro produced bovine embryos after vitrification and direct in straw rehydration, Anim. Reprod. Sci. 45 (1996) 191–200.
[28] P. Vanderzwalmen, G. Bertin, C.H. Debauche, V. Standaert, E. Van Roosendaal, M. Vandervost, N. Bollen, H. Zech, T. Mukaida, K. Takahashi, R. Schoysman, Births after vitrification at morula and blastocyst stages: effect of artificial reduction of the blastocoelic cavity before vitrification, Hum. Reprod. 17 (2002) 744–751.
[29] A. Van Soom, Y.Q. Yuan, L.J. Peelman, D.G. de Matos, J. Dewulf, H. Laevens, A. de Kruif, Prevalence of apoptosis and inner cell allocation in bovine embryos cultured under different oxygen tensions with or without cysteine addition, Theriogenology 57 (2002) 1453–1465.
[30] A.J. Watson, G.M. Kidder, Immunofluorescence assessment of the timing of appearance and cellular distribution of Na/K ATPase during mouse embryogenesis, Dev. Biol. 126 (1988) 80–90.
[31] C. Wrenzycki, D. Herrmann, L. Keskintepe, A. Martins, S. Sirisathien, B. Brackett, H. Niemann, Effects of culture system and protein supplementation on mRNA expression in pre-implantation bovine embryos, Hum. Reprod. 16 (2001) 893–901.