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Vitrification of in vitro produced ovine embryos at various developmental stages using two methods ☆

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ABSTRACT

This study was conducted to evaluate the effects of developmental stage of in vitro produced (IVP) ovine embryos and the type of vitrification procedure used on embryo cryotolerance.

The IVP embryos were vitrified at five different developmental stages: 4-, 8- and 16-cell, morula, and blastocyst. For each stage, half of the embryos were vitrified in either 30 μl 3.4 M glycerol + 4.6 M ethylene glycol in straw (method 1) or in <0.1 μl 2.7 M ethylene glycol + 2.1 M Me_2SO + 0.5 M sucrose placed on the inner surface of a straw (method 2) of vitrification solution, based on two different procedures. After warming embryo viability was determined by assessing the rates of re-expansion, survival, and blastocyst formation. The quality of surviving embryos was evaluated by their hatching rate and blastocyst cell numbers. In both vitrification methods, embryo survival progressively increased as the developmental stage progressed. In method 1 few of the early cleavage stage embryos (4-, 8- and 16-cell) could reach to the blastocyst stage following warming. There was no significant difference in blastocyst cell numbers (total, ICM, and trophectoderm cells) or hatching rate of blastocysts derived from vitrified embryos at different developmental stages. The number of dead cells in vitrified blastocysts in method 1 was higher than for non-vitrified blastocysts ($P < 0.05$). The number of apoptotic cells in vitrified blastocysts was higher than for non-vitrified counterparts ($P < 0.05$). In conclusion, both the developmental stage of IVP ovine embryos and the method of vitrification have a significant effect on the viability and developmental competence of sheep embryos.

Introduction

Cryopreservation of preimplantation embryos has progressed to become a useful adjunct to in vitro fertilization (IVF), allowing storage of those embryos excess to immediate requirements for the future use in human and animal embryo transfer programs.

The principal factors affecting successful cryopreservation of mammalian embryos are the species, the type, and concentration of cryoprotectants [61], cooling and warming rates [2] and also the developmental stage at which the embryos are cryopreserved. Moreover, the toxicity of cryoprotectants, the composition of the cryoprotectant solution [3,8], the length of exposure and the protein composition of the cryoprotectant medium affect embryo survival [35,40].

Among those factors, embryo developmental stage [5,28] is considered to be a critical factor for the viability of the embryo after cryopreservation. Additional concerns have been raised regarding the cryopreservation procedures that are used to preserve in vitro derived embryos, which show low cryotolerance compared with in vivo derived embryos [18,29]. Moreover, embryo survival of in vitro produced (IVP) embryos is affected by the choice of cryopreservation technique (vitrification vs slow cooling). Rapid cooling procedures (vitrification) provide a simple, economical and time-saving approach to cryopreservation compared with that of controlled rate slow cooling. The success of vitrification procedures are now known to be enhanced by using carriers with small volumes such as open pulled straws [56], superfine open pulled straws [21], cryoloops [7,10,17], and cryotops [23,24,26] which increase the cooling rate several fold in comparison to the traditional 0.25 ml straws. In some species early and late stage embryos have comparable cryopreservation outcomes. However, early embryos of sheep [1,50], cattle [33], and pig [6] are more sensitive to cryopreservation than embryos at advanced stages of development.

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Protocols which improve the cryopreservation outcome of early sheep embryos (2–4 cell stage of development) would limit the possible negative effects of in vitro culture [59], and potentially increase the developmental potential of embryos after transfer.

Relatively few studies have examined the effect of embryonic stage on cryotolerance of ovine embryos produced in vitro. The aim of the current study was therefore to assess the sensitivity of IVP ovine embryos to cryopreservation at different developmental stage using two different cryopreservation procedures.

Materials and methods

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

Oocyte collection

Sheep ovaries were collected from Shahrekord slaughterhouse (Latitude: 32′17″ N; Longitude 50′51″ E; Altitude 2049 m) and transported 30 km to the laboratory in saline (30–35 °C) in a thermos flask, within 1–3 h following collection. Ovaries were washed three times with prewarmed fresh saline (37 °C), and all visible follicles with a diameter of 2–6 mm were aspirated using gentle vacuum (30 mm Hg) via a 20 gauge short beveled needle connected to a vacuum pump. Prior to aspiration, the collecting tube was filled with 2 ml preincubated Hepes-modified TCM, supplemented with 50 IU/ml heparin.

In vitro maturation

After aspiration, only oocytes surrounded by more than three layers of unexpanded cumulus cells (COCs: cumulus oocyte complexes) were recovered and selected for in vitro maturation (IVM). Before culturing, oocytes were washed in Hepes-buffered TCM199 (H-TCM199) supplemented with 5% FBS (Fetal bovine serum, Gibco 10270), and 2 mM glutamine. The oocyte culture medium (OCM) consisted of bicarbonate-buffered TCM 199 with 2 mM L-glutamine supplemented with 0.02 mg/ml cysteamine, 1 IU/ml hCG, 1 μg/ml E_2, 100 μl/ml penicillin, 100 μg/ml streptomycin, 10% FBS (Fetal bovine serum, Gibco 10270), and 0.2 mM Na–Pyruvate. The medium osmolarity was adjusted to 275 mOsm. The COCs were randomly distributed in maturation droplets (10 oocytes in 50 μl) and covered by sterile paraffin oil in a 60-mm Petri dish (Falcon 1008; Becton Dickinson, Lincoln Park, NJ) and were then incubated under an atmosphere of 5% CO_2 −95% air with 100% humidity at 39 °C for 24 h.

Preparation of sperm and in vitro fertilization

Before transfer to fertilization drops, the oocytes were washed four times in H-SOF (Hepes-Synthetic Oviduct Fluid) and once in fertilization medium. The H-SOF and fertilization media were the same as used by Tervit et al. [52] with some modification as such for preparing H-SOF, 20 mM of $NaHCO_3$ was substituted with 20 mM Hepes (10 mM free acid and 10 mM Na salt). Both media were supplemented with antibiotics.

Fresh semen was collected from a Lori-Bakhtiari breed ram of proven fertility. For swim up, 80–100 μl of semen was kept under 1 ml of BSA-HSOF in a 15 ml conical Falcon tube at 39 °C for up to 45 min. After swim up, the 700–800 μl of the supernatant was added to 3 ml of BSA-HSOF, centrifuged twice at 200*g* for 3 min and the final pellet was resuspended with BSA-HSOF. Insemination was carried out by adding 1.0×10^6 sperm/ml to the fertilization medium. The fertilization medium was SOF enriched with 20% heated inactivated estrous sheep serum. A 5 μl aliquot of sperm suspension, containing 1×10^6 sperm/ml, was transferred into fertilization medium that included 10 oocytes per 45 μl fertilization drop. Fertilization was carried out by co-incubation of sperm and oocytes in an atmosphere of 5% CO_2 in humidified air at 39 °C for 22 h.

In vitro culture

After IVF, presumptive zygotes were vortexed for 2–3 min to remove the cumulus cells and then washed in H-SOF to remove spermatozoa and cellular debris. They were then allocated to 20 μl culture drops (five to six embryos/drop) consisting of SOF supplemented with 2% (v/v) BME-essential amino acids, 1% (v/v) MEM-nonessential amino acids, 1 mM glutamine and 8 mg/ml fatty acid free BSA. The incubation conditions were humidified 7% O_2, 5% CO_2, and 88% N_2 at 39 °C. On the third and fifth day of culture (Day 0 defined as the day of fertilization) 10% charcoal stripped fetal bovine serum (FBS) was added to the medium. The culture was continued until 8 days post-fertilization. The osmolarity was maintained at 270–285 mOsm.

Experimental design

In vitro produced ovine embryos were used to assess the possible effect of developmental stage of embryo and the method of vitrification used (in a 0.25 ml straw) on embryo survival after cryopreservation. The IVP embryos were distributed in five experimental groups according to the stage of development: 4-cell ($n = 64$), 8-cell ($n = 66$) and 16-cell ($n = 64$), morula ($n = 66$), and blastocyst ($n = 146$). The embryos in each developmental stage were randomly divided into two groups and each group was vitrified according to the procedure of either method. The two methods of vitrification was completely different from each other in term of basic, equilibration, and vitrification media as well as the procedure and the volume of vitrification solution (30 μl vs <0.1 μl). The vitrified embryos were warmed one week later and then cultured in vitro until the blastocyst and hatched blastocyst stages. Post warming the viability of embryos up to morula stage was assessed by their re-expansion, resumption of cellular division and reaching the blastocyst stage and for those that were vitrified at the blastocyst stage, by assessment of their re-expansion and hatching rate.

Embryo quality was assessed from hatching rates and the number of cells (total, ICM, trophectoderm, dead, and apoptotic cells) in the blastocysts produced in each experimental group after cryopreservation. The non-vitrified in vitro produced blastocysts were considered as controls for evaluation of the effect of cryopreservation on blastocyst cell numbers.

Vitrification and warming procedures

Method 1

In this method the embryos were vitrified according to Naitana et al. [38] with minor modification. Briefly, the basic media (PB1) for preparation of all vitrification solutions was prepared in Ca^{2+}–Mg^{2+} free PBS [32] supplemented by 0.3 mM sodium pyruvate, 3.3 mM glucose, 100 IU/ml penicillin, and 20% (v/v) FCS. The embryos were sequentially exposed to corresponding equilibration and vitrification solutions at room temperature. For equilibration of embryos up to the morula stage, the embryos were placed into a 100 μl drop of equilibration solution containing 1.4 M glycerol for 7 min, and then transferred to 100 μl of the second equilibration solution (1.4 M glycerol and 3.6 M ethylene glycol) for 3 min at 25 °C. The corresponding equilibration times for expanded blastocyst were 5 min for each equilibration drop. The embryos were then transferred to a column of vitrification solution (3.4 M

glycerol and 4.6 M ethylene glycol) at the centre of 0.25 ml straws using a fine glass capillary pipette. The column of vitrification solution (30 μl) in the straws was separated by 2 air bubbles from 2 columns of dilution (190 μl) solution (PB1 containing 1 M sucrose). The end of the straws were sealed and then plunged immediately into LN2 and stored until use. The total time limit for the exposure of embryos to the final vitrification solution and the immersion of straws into LN2 was 45 s. One week after vitrification embryo survival was evaluated by transferring the straws from LN2 to the air for 10 s followed by immersion into a water bath at 25–30 °C for 8 s. The straws were then shaken and the contents of each straw were expelled into a dilution solution containing 1 M sucrose at room temperature, the medium was stirred gently to facilitate the mixture of the two solutions and the embryos 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 (two times) in PB1. The embryos were then cultured in IVC-SOF medium until the blastocyst and hatched blastocyst stage.

Method 2

In this method the embryos were vitrified according to Chian et al. [4] with minor modifications. Briefly, the basic media for preparation of all vitrification solutions was DMEM supplemented by 5.5 mM glucose, 19 mM $NaHCO_3$, 25 mM Hepes, 100 IU/ml penicillin, and 20% (v/v) FCS. All equilibration and dilution steps as well as warming were performed at room temperature (approximately 25 °C).

The embryos were placed into a 100 μl drop of basic medium (20–30 s) and were then transferred to the equilibration medium. For the first equilibration step of embryos up to the morula stage were placed into a 100 μl drop of equilibration solution (1.35 M ethylene glycol + 1.05 M Me_2SO) for 5 min, and then transferred to a 100 μl drop of vitrification solution (2.7 M ethylene glycol + 2.1 M Me_2SO + 0.5 M sucrose) for 2 min. The corresponding equilibration times for expanded blastocyst were 8 min and 30 s for equilibration and vitrification solutions, respectively. The embryos were then loaded with a fine bore pasture pipette onto the inner surface of the tip of sharpened 0.25 ml straw (the straw was cut at an angle with the scalpel blade to resemble the tip of long beveled injection needle) with a the minimum volume of vitrification medium (<0.1 μl). After loading, almost all the solution was removed with a fine bore pasture pipette and the straw was quickly immersed in liquid nitrogen.

The time limit from the time the embryos was being transferred to the tip of straw until the immersion of straws into LN2 was 45 s. For warming, the tip of straw was directly immersed into the 100 μl drop of dilution solution containing 0.5 M sucrose for 5 min and then washed (two times) in basic medium. The cryopreserved embryos were cultured in IVC-SOF medium until blastocyst and hatched blastocyst stage.

Cell counting and TUNEL assay

Blastocysts were washed in PBS supplemented with 0.1% PVP and then incubated in 30 μg/ml propidium iodide (PI) prepared in base medium (H-SOF containing 5 mg/ml BSA) for 3 min at 39 °C. 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 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 the inner cell mass (ICM) and TE cell 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 followed by two washes in the base medium. The blastocysts were then transferred into ice-cold ethanol containing 10 μg/ml Hoechst 33,342 for 15 min. The blastocysts were directly mounted into a small droplet of glycerol on a glass slide and examined under an epifluorescent microscope. ICM nuclei appeared blue, caused by DNA labeling with Hoechst 33342, while TE cells appeared red due to staining of nuclear DNA with the membrane impermeable PI.

For counting the number of cells with DNA-fragmented nuclei (apoptotic cells), the stained blastocysts were then fixed in 4% (v/v) paraformaldehyde prepared in PBS supplemented with 0.1% PVP (PBS-PVP) for 2 h at room temperature. Membranes were permeabilized on ice in 0.1% Triton X-100 (0.1% sodium citrate in PBS) for 1 h at room temperature. Following two more rinses in PBS-PVP, the permeabilized blastocysts were incubated in microdrops of the fluorescein-conjugated dUTP and TdT (In situ cell detection kit, fluorescein, Roche Diagnostics, Germany), for 1 h under oil in a humidified atmosphere at 38 °C in the dark. The reaction was stopped by transferring the blastocysts into PBS supplemented with BSA (0.5% (w/v)) for 10 min in the dark. The blastocysts were then incubated in 0.1 mg/ml RNase A (from bovine pancreas) solution.

The embryos were washed twice in BSA-PVP and then mounted in a small drop of glycerol on a glass slide under coverslip compression and examined under an epifluorescent microscope. The embryos treated with DNAse before TUNEL staining served as a positive control for TUNEL labeling; for a negative control the terminal transferase enzyme was omitted during TUNEL labeling. The DNA-fragmented nuclei in apoptotic cells appeared green.

Statistical analysis

Data was collected over at least five replicates. The blastocyst cell numbers were analyzed using one-way ANOVA. When ANOVA revealed a significant effect, the experimental groups were compared by Tukey method. When equal variance test was failed the treatments were compared by Student–Newman–Keuls Method. When normality test failed the Kruskal–Wallis One Way Analysis of Variance on Ranks was applied. Chi-square and Fisher Exact Test was applied when qualitative evaluation was considered. A $P < 0.05$ level was considered significant (Sigma Stat Ver.2).

Results

The proportion of ovine embryos that survived and developed further after cryopreservation was influenced by the developmental stage of embryos as well as the cryopreservation method. With both methods survival was highest proportion when embryos were vitrified at the blastocyst stage. Of the two protocols, method 2 achieved higher survival and re-expansion rates compared to method 1 for all stages except for the blastocyst stage (Table 1).

The embryos vitrified at the 4-, 8- and 16-cell stages, using method 1, did not reach the blastocyst stage after cryopreservation. In embryos which had been vitrified by method 2, the highest blastocyst formation rate was seen in those vitrified at the morula stage.

Method 1 had a lower blastocyst percentage for 4-, 8- and 16-cell embryos as compared with corresponding values in controls.

The highest rate of hatched blastocyst was achieved in those vitrified at the blastocyst stage regardless of method (Table 1). In both methods, there was no significant difference in hatching rate between cryopreserved embryos at different stages and corresponding values in controls.

There was no significant difference in blastocyst cell numbers (total, ICM, and trophectoderm cells) among blastocysts derived from embryos which had been cryopreserved at different embryonic

Table 1
Post-warming development of vitrified ovine embryos using two different methods at various embryonic stages.

Embryonic stage	Vitrification[1]	No. of embryos		Re-expansion *n*(%)		Survival *n*(%)		Blastocyst *n*(%)		Hatched blastocyst[2] *n*(%)	
		M1	M2	M1	M2	M1	M2	M1	M2	M1	M2
4-Cell	+	31	33	5(16.1)^{a,A}	15(42.9)^{a,B}	1(3.2)^{a}	5(15.1)^{a}	*0(0)^{a}	*1(3)^{a}	0(0)	0(0)
	–	35	35	–	–	–	–	**19(54)	**19(54)	16(84)	16(84)
8-Cell	+	27	39	6(22.2)^{a,A}	20(51.3)^{a,b,B}	3(11.1)^{a,b,A}	15(38.5)^{a,b,B}	*0(0)^{a,A}	*7(17.9)^{a,b,B}	0(0)	6(85.7)
	–	35	35	–	–	–	–	**17(49)	**17(49)	13(76)	13(76)
16-Cell	+	29	35	11(37.9)^{a}	22(62.9)^{a,b}	5(17.2)^{a,b,A}	16(45.7)^{b,c,B}	*0(0)^{a,A}	10(28.6)^{b,c,B}	0(0)	7(70)
	–	31	31	–	–	–	–	**12(39)	12(39)	10(83)	10(83)
Morula	+	36	30	14(38.8)^{a,A}	23(76.7)^{b,c,B}	12(33.3)^{b,A}	20(66.7)^{c,d,B}	10(27.7)^{b}	16(53.3)^{c}	6(60)	11(68.7)
	–	32	32	–	–	–	–	13(41)	13(41)	10(77)	10(77)
Blastocyst	+	92	54	81(88)^{b}	44(81.5)^{c}	81(81)^{c}	44(81.5)^{d}	–	–	69(85.2)	38(86.4)
	–	61	61	–	–	–	–	–	–	50(82)	50(82)

*,** For each embryonic stage the values with different asterisk in the same column differ significantly ($P < 0.01$).
$^{a–d}$Numbers with different lowercase superscript in the same column differ significantly ($P < 0.05$).
^{A,B}Numbers with different uppercase superscript in the same row of each parameter differ significantly ($P < 0.05$).
M1, method 1; M2, method 2.
[1] The number of non-vitrified embryos and related blastocyst and hatched blastocyst rates has been repeated for each method, as control, in order to compare with corresponding values in cryopreserved embryos.
[2] The proportion of hatched blastocysts was expressed on the basis of number of blastocysts.

stages using different methods and the non cryopreserved controls. The number of dead cells was significantly higher when embryos had been vitrified at the blastocyst stage, using method 1 (30 μl in straw), compared with control or method 2 (<0.1 μl) (Table 2).

The number of apoptotic cells in embryos vitrified at the blastocyst stage was significantly higher than for non-vitrified counterparts regardless of the method used.

Discussion

Data analysis showed that the developmental stage of the ovine embryos produced in vitro affected their survival and blastocyst formation rates after vitrification and warming regardless of the method used ($P < 0.05$). Survival following vitrification of IVP embryos progressively increased as the developmental stage proceeded. Among early stage embryos produced in vitro, those cultured to the blastocyst stage before cryopreservation had the highest rate of survival rate after warming. However, there was no significant difference in hatching rate of blastocysts derived from cryopreserved early stage and more advanced stage embryos as well as their corresponding controls. This finding was in contrast to a report indicating that cryopreserved early stage in vivo derived sheep embryos were able to reach the blastocyst stage during the in vitro culture, but failed to hatch from the zona pellucida [13]. Our data was in agreement with previous studies indicating that developmental stage influences the cryotolerance of in vivo produced sheep and cattle embryos; early developing stages would have a high sensitivity to cooling [5,33].

In the current study the survival rates of sheep embryos vitrified in small volumes (<0.1 μl) at early stages were comparable to those reported in cryopreserved in vivo derived ovine early embryos using 0.25 ml straw [13]. As shown the increase in cryosurvival rates with more advanced stages may in part be related to the higher number and the smaller size of cells [37,62], which improve chilling tolerance when compared to the early stage embryos with few and large cells [48]. The larger cells in early stage embryos than the cells of blastocysts, may render them more sensitive to the osmotic stress induced by the removal of the permeating cryoprotectant during the dilution procedure [51]. During vitrification, embryo exposure to a highly-concentrated solution of cryoprotectants can produce irreversible damage to the cytoskeletal organization of embryos, in particular at the earlier stages [38,41]. Moreover, it seems that the decrease in lipid content of embryonic cells in more advanced stage embryos would be another reason for their higher cryotolerance compared to the early stage embryos [36,53]. Whether embryo genomic activation after 9–12 cell stage in sheep embryo could increase the cryotolerance of embryos, should be further investigated. In this context, there is a report noting that the cryosurvival of in vivo derived 9- to 12-cell sheep embryos was lower than that of 5- to 8-cell. The authors have proposed that the difference, though insignificant, might be related to the maternal embryonic transition of genetic control of the development [1,14].

Cooling rate is another key factor in embryo vitrification. More rapid cooling and warming reduces membrane damage and leakage [20]. An increase in cooling rate decreases chilling injury (lateral segregation of proteins and phospholipids within membranes) [10,20,43,46] by rapidly passing the certain critical temperature zone of 15 to −5 °C (10). Furthermore, by increasing cooling and warming rates, it is possible to reduce the cryoprotectant concentration and, thus the toxic and osmotic effects of them are decreased [55].

Some other aspects of chilling injury such as protein denaturation and uncoupling of ATP synthesis from electron transport chain mitochondrial enzymes could also be reduced by rapid cooling [44].

One approach for increasing the cooling rate is minimizing the amount of medium surrounding embryos at the time of cooling. In the current study, the rates of survival and blastocyst formation in cryopreserved early stage embryos up to morula stage were best with method 2. In this context, it is documented that to facilitate vitrification by even higher cooling rates, it is necessary to minimize the volume of the vitrification solution as much as practical [32,34,42,57]. For instance, the 0.25 ml volume straws limit the cooling rate to less than 2500 °C/min [43]. While vitrification by using carriers such as the cryotop, increase the cooling rate approximately ninefold (22,800 °C/min) in comparison with the 0.25 ml straws. Likewise, the warming rate is 42,100 and 1300 °C/min for cryotops and straws, respectively [25]. With the cryotop almost all medium surrounding the embryos was removed before cooling procedure as the remaining fluid surrounding embryos is less than 0.1 μl. Therefore, the risks of chilling injuries using small volumes in method 2 are lower than for large volumes (30 μl) in method 1 [25,31].

This study did not investigate whether the differing outcomes of the two tested methods were caused only by the vitrification volume or to the other differences between the two procedures. Differences included cryoprotectant type, composition and

Table 2
Effect of vitrification method on cell numbers of blastocyst-derived ovine embryos vitrified at different stage of development.

Experimental groups	Embryonic stage	No. of blastocysts		Embryonic cells (mean ± SEM)(%)									
				Total		ICM		Trophectoderm		Dead cells		Apoptotic cells	
		M1	M2	M1	M2	M1	M2	M1	M2	M1	M2	M1	M2
Non-vitrified*	Blastocyst	30	30	147.9 ± 9.4	147.9 ± 9.4	31.6 ± 2.6	31.6 ± 2.6	121.4 ± 9	121.4 ± 9	4.7 ± 0.5(3)[a]	4.7 ± 0.5(3)	4.6 ± 0.3(3)[a]	4.6 ± 0.3(3)[a]
Vitrified	8-Cell	0	7	–	139.2 ± 21.9	–	28.7 ± 4.8	–	110.5 ± 18.2	–	6.2 ± 1.7(4)	–	NA
	16-Cell	0	10	–	130.0 ± 7	–	25.0 ± 3.4	–	105.0 ± 6.5	–	5.0 ± 0.9(4)	–	NA
	Morula	10	16	139.7 ± 12.4	144.4 ± 11	27.7 ± 3.7	29.4 ± 4.0	112.1 ± 10.4	115.0 ± 7.9	7.5 ± 1(5)[a]	5.8 ± 0.9(4)	NA	NA
	Blastocyst	15	15	139.2 ± 10.8	139.6 ± 7.1	25.3 ± 2.1	23.5 ± 2.3	120.5 ± 11.1	116.1 ± 6.6	22.4 ± 2.7(16)[b,A]	9.7 ± 1.9(7)[B]	17.3 ± 0.9(12)[b]	3.9 ± 1.3(10)[b]

[a,b]Numbers with different lowercase superscript in the same column differ significantly ($P < 0.05$).
[A,B]Numbers with different uppercase superscript in the same row of each parameter differ significantly ($P < 0.05$).
M1, method 1; M2, method 2.
* In this group the total number of blastocysts and blastocyst cells allocations has been repeated for each method of vitrification as a control in order to compare with corresponding numbers in cryopreserved embryos.

equilibration procedures. The presence of sucrose in the vitrification solution used in method 2 may have contributed. It has been reported that hypertonic solutions in the range of 1.2–1.5 times isotonicity could abolish chilling injury between 0 and −22 °C [11]. Therefore, the presence of sucrose in the vitrification solution used in method 2 may have had an effect by increasing the tonicity of the vitrification solution or the degree of dehydration as sucrose does not readily permeate the membrane and acts as an osmotic counterforce to water and cryoprotectant movement.

In sheep, a good survival rate has been obtained after reducing the total time of exposure to the vitrification solution at low temperatures [49,50]. Moreover, toxicity of high concentrations of cryoprotectants during equilibration will be decreased by reducing the equilibration period. In current study the equilibration time for method 2 was shorter than with method 1 (7–8.5 min vs 10 min). Meanwhile, in method 1 the longer equilibration procedures and the presence of more cryoprotectant after warming both increases the risk of embryo exposure to toxic effect of cryoprotectants. Furthermore, the longer exposure of embryos to cryoprotectants can have indirect consequences, such as biochemical injuries due to specific interactions between the cryoprotectant and proteins, lipids, or DNA, modifying the transport systems [54], irreversible damage to the cytoskeletal organization of embryos [9], and the impairment of embryo ability to regulate pH [27], with consequences to the perturbations of metabolism [47], glucose uptake and lactate production [15].

The higher cryotolerance of vitrified in vivo derived ovine and porcine expanded blastocysts compared with morula and early stage embryos [6,38] confirmed the results of the current study indicating that the cryotolerance of IVP embryos increased as the developmental stage of embryos progressed. The higher cryotolerance of ovine blastocyst compared with those at earlier stages might be related to the higher resistance of their cellular membranes to osmotic and toxic stress after the formation of the blastocoelic cavity [38]. As documented the increase of Na/K ATPase activity which occurs during blastocoelic formation in trophoblastic cells [61] may determine more active transport mechanisms of cryoprotectants leading to decrease exposure time and low concentration of cryoprotectants needed during cryopreservation [61]. Furthermore, the blastomere of the blastocyst have a higher surface area to volume ratio than early stage embryos which may contribute to the former to having higher permeability coefficient to water and cryoprotectant than the latter [37,38]. Therefore, blastocysts are more tolerant to osmotic stress than early stage embryos.

The use of highly permeable cryoprotectants such as ethylene glycol, has been suggested to reduce the osmotic damage at warming [58,60] even in early stage embryos. In the rat, 2-cell embryos appear to be less permeable to ethylene glycol than 4-, 8-cell, and morula stage embryos, confirming the higher permeability of more advanced embryos compared with earlier stage embryos [19].

Another explanation for the higher cryotolerance of the blastocyst stage embryos, apart from their higher cell numbers would be related to the higher proportion of nucleus to cytoplasm. Finally, the increased cryotolerance at the latter stage may be due to prior selection. Indeed, developmentally compromised or incompetent embryos may be lost during early development in vitro, such that the latter stages represent a selected group of more competent embryos.

None of the early stage embryos (4-, 8- and 16-cell stages) vitrified by using method 1 reached the blastocyst stage after warming. While in those vitrified by using method 2 the proportion of embryos which reached the blastocyst stage after warming with method 1 ranged from 3% to 53.3% for the 4-cell and morula stages, respectively. Additionally, the blastocyst rate in cryopreserved embryos at 16-cell stage in method 2 and those cryopreserved at the

morula stage in both methods were comparable with the corresponding values in the control groups.

No significant difference in blastocyst quality was observed among embryos cryopreserved at different developmental stages by using either method in terms of hatching rate. There was also no significant difference in blastocyst cell numbers (total, ICM, and trophectoderm cells) among blastocysts derived from cryopreserved early stage and more advanced embryos as well as non-vitrified blastocysts. Moreover, the blastocysts cell numbers were not affected by the method of cryopreservation.

Cell death is a normal process but can be increased by vitrification [45]. In the current study the number of dead cells in the blastocysts derived from cryopreserved early and more advanced embryos was affected by the cryopreservation procedure and the method of vitrification. Indeed, in embryos vitrified by using method 2 (modified 0.25 ml straws), no significant difference was observed in the number of dead cells between vitrified and non-vitrified embryos. The number of dead cells in cryopreserved blastocysts was significantly higher in method 1 than method 2 which correlates with the differences in embryo development seen with these two methods.

The cryopreserved embryo needs time to fully restore the biological metabolism, which diminishes at low temperatures [12] and repair the structural and metabolic damage due to cryopreservation procedures [9,16,22]. In current study the lower proportion of dead cells in blastocysts derived from cryopreserved morula stage embryos compared with those vitrified at blastocyst stage by using method 1 (5% vs 16%) could be related to the longer culture period after warming in the former. It has been reported that the resumption of DNA synthesis and normal secretory activities in vitrified/warmed ovine embryos resume after 9–12 and 29–35 h, respectively [30]. Therefore, the longer culture period was provided more time for cellular regeneration especially in the cryopreserved morula stage embryos compared with those vitrified at blastocyst stage could have contributed to the lower proportion of dead cells in the former.

The proportion of dead cells in blastocysts derived from embryos cryopreserved at different developmental stage by method 2 (5–9.7%) was comparable to the rate (9–10%) reported for blastocysts derived from cryopreserved in vivo derived embryos [39]. There was no significant difference in the number of apoptotic cells in blastocysts cryopreserved with either method but both were significantly higher compared with non-vitrified counterparts.

In conclusion, early stage IVP-sheep embryos were more sensitive to either cryopreservation method than more advanced embryos with the survival rates and subsequent embryo development following cryopreservation improved as the embryo developmental stage progressed. However, the reason for the differences between the two tested methods on the IVP-sheep embryos was not determined.

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