

In vitro culture of in vivo Saanen goat embryos by vitrification

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Abstract: The purpose of this study was to investigate the survival rate of high-quality embryos (n: 101) derived in vivo from Saanen goats (n: 15) in culture following their vitrified freezing and thawing during the breeding season. The in vitro postthaw survival rates of 101 vitrified embryos at 24, 48, and 72 h were 59.4%, 33.6%, and 25.7%, respectively. According to the developmental stage of the embryos, the survival rates were 51.3%, 20.5%, and 15.4% in the morula, and 64.5%, 41.9%, and 32.3% in the blastocyst, respectively. The survival rates of the blastocysts and morulae at 48 and 72 h were significantly different ($P < 0.05$). While the survival rates of the embryos of Grade 1 quality at 24, 48, and 72 h were 78.6%, 46.4%, and 32.1%, Grade 2 quality survival rates were 35.5%, 17.8%, and 17.8%, respectively. The survival rates of Grades 1 and 2 embryos at 24 h ($P < 0.001$) and 48 h ($P < 0.05$) were significantly different. As a result, it was concluded that the embryos to be frozen should be selected according to their development stage and quality, and preferably should be of Grade 1 quality in the blastocyst stage for the cryopreservation of in vivo-derived goat embryos by vitrification.

Key words: Embryo, goat, in vitro culture, vitrification

1. Introduction

The freezing of embryos was first reported in the 1970s (1). In the livestock sector, embryo cryopreservation enables the protection of endangered animal species and breeds, preservation, and improvement of fertility at lower cost through the establishment of embryo banks. Furthermore, the transfer of frozen embryos enables the establishment of herds with high production yields, control of diseases, and long-term preservation of genetic material (2–5). Embryo cryopreservation aims at preserving embryos in their existing state and enabling their development after being thawed. When preserved at low temperatures, intracellular enzyme activity, cell respiration, metabolism, development, and cleavage of embryos cease. Thus, the embryo can be preserved without harming its long-term survival and causing any genetic damage (1,6,7).

Currently available methods used for embryo cryopreservation are classified under three groups: conventional freezing (slow freezing), rapid freezing, and vitrification (7,8). The vitrification process is the freezing of embryos in a glass-like state, in concentrated vitrification solutions containing high concentrations of cryoprotectants ($>40\%$ = approximately 6–8 M), by applying rapid cooling rates to prevent ice crystal formation (7,9–12). Vitrification has been used as an alternative to conventional cryopreservation for a long time (10,13,14).

This vitrification method has several advantages, including ease of application, low cost, rapid results, no need for specific equipment, easy adaptation to routine use, and the prevention of ice crystal formation during the freezing of the embryo (6,15–18). Cryoprotectants such as glycerol, ethylene glycol, and propanediol are used alone in the conventional freezing method for embryo cryopreservation, whereas the vitrification method involves the use of cryoprotectant combinations such as glycerol + ethylene glycol and glycerol + propanediol (19,20).

For the minimisation of the toxic effects of cryoprotectants in the vitrification method, it is required that the equilibration period is shortened, stepwise (two-step) equilibration is performed, and toxicity is reduced by the use of nonpermeating agents such as sucrose, trehalose, and formamide (6,7,19). It has been reported that the most appropriate embryonic development stage for use in vitrification is the blastocyst (3,7,21–23). It was reported that the survival rate of embryos in the blastocyst stage is higher than those in the morula stage in cryopreservation with slow freezing or vitrification (19,24).

The purpose of this study was to investigate the survival rate of high-quality embryos derived in vivo from Saanen goats in culture media following their vitrification and thawing during the breeding season.

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2. Materials and methods

2.1. Study material

This study was conducted during goat breeding season (October–November) in the Research and Practice Farm of Selçuk University, College of Veterinary Medicine located at 36°52'N, 32°29'E.

All the experimental procedures followed in this study were approved by the Local Ethics Board for Animal Experiments (SÜVFEK; 2011/045). Donors and bucks used in this study were, respectively, 15 female and 2 male healthy Saanen goats, between 2 and 3 years old, weighing on average 40–65 kg, with no reported reproductive problems. These animals were fed high alfalfa and concentrate feed (in pellet form) and were provided with water ad libitum.

2.2. Oestrous synchronisation and superovulation

All goats were progesterone impregnated (20 mg of flugestone acetate; Chronogest CR, Intervet, Beaucauzé, France) with intravaginal sponges, irrespective of the stage of the oestrous cycle, for oestrous synchronisation during the breeding season. The intravaginal sponges were applied for a period of 11 days. Twenty-four hours after the removal of the intravaginal sponges, the goats in oestrous were detected by teaser goats. The day of the intravaginal sponge application is accepted as day 0, and by day 9, for superovulation, the goats received, via intramuscular (IM) route, decreasing doses of follicle-stimulating hormone (FSH) (2.5 mL; 1.5 mL; 1 mL), twice a day in the morning and evening for a period of 3 days, such that each animal received total of 200 mg of FSH (400 mg/20 mL NIH-FSH-P1; Folltropin-V, Bioniche, Inervin, Ireland). On the first day of FSH administration, 125 µg of prostaglandin F2α (PGF2α) (263 µg/1 mL cloprostenol sodium; Estrumate, Intervet, Unterschleibetaheim, Germany) was also administered by IM route in the morning. On day 11 of the protocol, the intravaginal sponges were removed and on day 12, 4 µg of gonadotropin-releasing hormone (GnRH) (0.0042 mg/mL buserelin acetate; Receptal, Intervet, Germany) was injected by IM route in the morning. Four hours after the injection of GnRH, controlled natural mating was performed twice, once in the morning and evening (25). Oestrous synchronisation and superovulation were performed twice, in different breeding seasons.

2.3. Embryo recovery and assessment

The operations to recover embryos were performed through uterine flushing on the goats on the seventh day following the first mating. Prior to the flushing of the uterine horns, all the goats were fasted for 12 h. Laparotomy was performed under general anaesthesia. Prior to anaesthesia, the goats were sedated with 0.22 mg/kg xylazine (23.3 mg/mL xylazine hydrochloride; Xylazinbio 2%, Bioveta,

Ivanovice na Hané, Czech Republic). For the induction of general anaesthesia, 2 mg/kg ketamine HCl (100 mg/mL ketamine hydrochloride; Ketazol 10%, Richter Pharma, Wels, Austria) was administered by IM route. Uterine flushing was performed using the conventional method, by laparotomy. For embryo collection, both uterine horns were flushed with tissue culture medium 199 (TCM 199) (500 mL; M 7528, Sigma, St. Louis, MO, USA) with 1% foetal calf serum (FCS) (500 mL; C8056, FCS, Sigma). The resulting effluents were immersed in a 37 °C water bath for 30–45 min for sedimentation of the embryos. Subsequently, the effluents were transferred into sterile petri dishes (100 × 20 mm; tissue culture petri dish, BD Falcon, Franklin Lakes, NJ, USA) and examined under a stereomicroscope (Olympus SXZ 16; Tokyo, Japan) for embryo detection. The detected embryos were transferred to Dulbecco's modified phosphate-buffered saline (mD-PBS) solution containing 1% FCS and 1% antibiotic (penicillin 10,000 IU/mL).

The goat embryos were scored for developmental stage and quality following the recommendations of the International Embryo Transfer Society. Accordingly, the embryos were classified as unfertilised oocyte, compact morula, early blastocyst, blastocyst, and expanded blastocyst according to their developmental stage. Furthermore, the embryos were scored as Grade 1 (excellent), Grade 2 (good), Grade 3 (poor), and Grade 4 (dead or degenerated) according to their morphological structure.

2.4. Embryo cryopreservation

The embryos of Grade 1 and Grade 2 quality were frozen by vitrification. Equilibration solutions (VS1 and VS2) and a vitrification solution (VS3) were used in the vitrification process. VS1 contained 20% ethylene glycol (E 9129; Sigma), and VS2 contained 20% ethylene glycol + 10% glycerol (G7757; Sigma). On the other hand, VS3 contained 25% ethylene glycol + 25% glycerol. The embryos were kept in VS1 and VS2 for 5 min each and in VS3 for 30 s. Subsequently, the embryos, together with their vitrification medium (VS3), were loaded into plastic embryo straws containing sucrose solution and air (0.25 mL, 133 mm straw; REF: 19040/0010, Minitube, Tiefenbach, Germany). After they were loaded again with air and sucrose solution following the transfer of the embryos, the straws were sealed. The straws were kept in liquid nitrogen vapour for nearly 10 s before they were plunged into liquid nitrogen at an angle of 45°. Once all the embryos were frozen, they were transferred into a nitrogen tank (Alta Genetik, MVE XC 47/11-10).

2.5. Embryo thawing

The straws were first exposed to room temperature for 10 s and then immersed in a 30 °C water bath for 20 s to thaw the frozen embryos. Next, the straws were wiped

with alcohol-dipped cotton and dried. The straws were waved in the air, and then cut at their sealed end before being transferred to empty sterile petri dishes preheated to 37–38.5 °C. The embryos were kept first in a mD-PBS + 20%FCS + antibiotic + 0.5 M sucrose solution and then in a mD-PBS + 20% FCS + antibiotic + 0.25 M sucrose solution for 5 min each with the aid of a stereomicroscope. After being kept in an mD-PBS + 20% FCS + antibiotic solution for a further 5 min, the embryos were washed. Subsequently, the embryos were transferred to the culture medium, which was prepared in the form of mineral-oil-coated drops (20% FCS + 0.1 mM βME+ TCM 199 + antibiotic), and were maintained in an incubator containing 5% CO₂ at 38.5 °C (SANYO MCO-18AC). The survival and development of the embryos were checked at 24, 48, and 72 h. The survival rates of the embryos were assessed on the basis of their developmental stage in the culture medium at 24, 48, and 72 h postthawing. The development of a morula into a blastocyst, followed by an expanded blastocyst and a hatching or hatched blastocyst, was considered an indicator of the viability of the embryo (Figure).

2.6. Statistical analysis

The statistical comparison of the in vitro survival rates of the embryos in culture media at 24, 48, and 72 h was made using the chi-square test (SPSS 11.0; Chicago, IL, USA).

3. Results

Effluents, collected by uterine flushing performed by laparotomy during the first and second breeding seasons of the goats, were examined under a stereomicroscope to reveal the recovery of 170 embryos and similar structures, which were classified on the basis of developmental stage and morphological structure. Of these structures recovered from the goats, 45 were classified as unfertilised ova, 24 as degenerate embryos, and 101 as freezable embryos (Table 1). Of the 101 freezable embryos, 39 were classified as compact morulae (38.6%) and 62 as blastocysts (61.4%) on the basis of their morphological structure. Fifty-six (55.4%) were determined to be of Grade 1 quality and 45 (44.6%) of Grade 2 quality (Table 1). All the freezable embryos were frozen by vitrification.

The in vitro survival rates of the 101 vitrified and thawed embryos at 24, 48, and 72 h in the culture media are presented in Table 2. Accordingly, it was observed that the survival rate of the embryos at 24 h was significantly higher than those at 48 and 72 h (P < 0.001, Table 2).

The in vitro survival rates determined for the 101 vitrified and thawed embryos, according to their developmental stage at 24, 48, and 72 h in the culture media, are presented in Table 3. Accordingly, the survival rates of the morulae and blastocysts at 24 h were significantly higher than those at 48 and 72 h (P < 0.05,

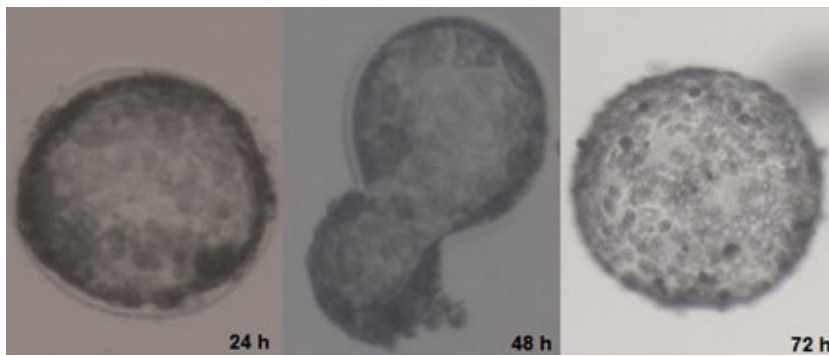


Figure. The postthaw appearance of embryos in culture for 24, 48, and 72 h.

Table 1. The number and stage of embryos obtained after uterine flushing.

UFO (n)	Degenerated embryo (n)	Freezable embryo (n)								Total (n)
45	24	101								170
		Developmental stage				Morphological grading				
		KM		B		G1		G2		
		n	%	n	%	n	%	n	%	
		39	38.6	62	61.4	56	55.4	45	44.6	

UFO: unfertilized ova, KM: compact morula, B: blastocyst, G1: Grade 1, G2: Grade 2.

Table 2. The survival rates of embryos following culture for 24, 48, and 72 h.

Culture period	24 h (%)	n	48 h (%)	n	72 h (%)	n
Embryos alive	59.4 ^a	60/101	33.6 ^b	34/101	25.7 ^b	26/101
χ^2	0.000					

^{a,b}: Groups with different letters in the same row are different from each other.

Table 3. The survival rates of the embryos in morula and blastocyst stages following culture for 24, 48, and 72 h.

Culture period	24 h (%)	(n)	48 h (%)	(n)	72 h (%)	(n)	P
Morula	51.3 ^a	20/39	20.5 ^b	8/39	15.4 ^b	6/39	0.001
Blastocyst	64.5 ^a	40/62	41.9 ^b	26/62	32.3 ^b	20/62	0.001
χ^2	0.133		0.021		0.042		

^{a,b}: Groups with different letters in the same row are different from each other.

Table 3). The comparison of the in vitro survival rates of the embryos, which were in the blastocyst and morula stages, in the culture media demonstrated that while there was no difference for the survival rates determined at 24 h ($P > 0.05$), the survival rates of the blastocysts were significantly higher than those of the morulae at 48 and 72 h ($P < 0.05$, Table 3).

The in vitro survival rates determined for the 101 vitrified and thawed embryos, according to their morphological structure at 24, 48, and 72 h in the culture media, are presented in Table 4. The in vitro survival rate of the embryos of Grade 1 quality at 24 h was significantly higher than that at 48 and 72 h ($P < 0.001$). On the other hand, no statistically significant difference was determined between the survival rates of Grade 2 embryos determined at these time periods ($P > 0.05$, Table 4). The comparison of the in vitro survival rates of Grade 1 embryos in culture media with those of Grade 2 embryos showed statistically significant differences in survival rates at 24 h ($P < 0.001$) and 48 h ($P < 0.05$), but not at 72 h ($P > 0.05$, Table 4).

4. Discussion

When using the conventional freezing method for the cryopreservation of embryos, most cryoprotectants, such as glycerol, ethylene glycol, and propanediol, are used alone, whereas fast freezing and vitrification involve the use of cryoprotectant combinations (19,20). Rao et al. (26) reported survival rates of 69% and 79% for the rapid freezing of goat embryos with glycerol and dimethyl sulfoxide (DMSO), respectively. Using different cryoprotectants for embryos in the expanded blastocyst stage in a vitrification study, a survival rate of 90.2% was obtained at 24 h with the use of 16.5% EG + 16.5% DMSO, whereas 83.5% and 64.7% were achieved at 24 h with the use of 20% EG + 20% DMSO and 12.5% EG + 12.5% DMSO + 8% 1,3 butanediol, respectively (27).

In the present study, the postthaw survival rates of the 101 embryos vitrified at 24 h were higher than those at 48 and 72 h, and the embryos started to degenerate at 48 and 72 h. The differences between the survival rates determined in the present study and those reported can be attributed to several factors, such as the utilisation of

Table 4. The survival rates of Grade 1 and Grade 2 embryos after culture for 24, 48, and 72 h.

Culture period	24 h (%)	(n)	48 h (%)	(n)	72 h (%)	(n)	P
Grade 1	78.6 ^a	44/56	46.4 ^b	26/56	32.1 ^b	18/56	0.000
Grade 2	35.5	16/45	17.8	8/45	17.8	8/45	0.073
χ^2	0.000		0.002		0.078		

^{a,b}: Groups with different letters in the same row are different from each other.

different cryoprotectants, using the frozen embryos in different developmental stages, keeping the embryos in equilibration and vitrification solutions for different time periods during the freezing process, and the difference of the volume of the vitrification solution that the embryos were held in during the transfer of the embryos to the straws.

It has been reported that the embryonic development stage most appropriate for use in vitrification is the blastocyst (3,22,23). Furthermore, reports indicate that the postfreezing survival rate of in vitro-produced embryos depends mostly on the developmental stage of the embryo rather than the protocol day. Results obtained so far suggest that among embryos frozen by slow freezing, rapid freezing, or vitrification, those in the blastocyst stage display better survival rates than those in the morula stage (19). It has been reported that for goat embryos vitrified with ethylene glycol and glycerol, the survival rate at 48 h in culture media was 23% for morulae and 45% for blastocysts when ethylene glycol was used, and 0% for morulae and 35% for blastocysts when glycerol was used (28). Martemucci and D'Alessandro (29) reported that, with the use of a three-step vitrification method based on the use of increasing doses of ethylene glycol and glycerol, the postthaw survival rate of in vivo-derived goat embryos was 77.4% for blastocysts and 66.6% for morulae. Huang et al. (27) reported that the postthaw survival rate of embryos, which were frozen with 16.5% EG + 16.5% DMSO at 24 h in the culture medium, was 80% for those in the morula stage and 88.8% for those in the blastocyst stage.

In the present study, the postthaw survival rates of the embryos at 24, 48, and 72 h were 51.3%, 20.5%, and 15.4%, respectively, for those in the morula stage, and 64.5%, 41.9%, and 32.3%, respectively, for those in the blastocyst stage. The survival rates of the blastocysts at 48 and 72 h significantly ($P < 0.05$) differed from the survival rates of the morulae at the same time periods. Similar to previous reports, these results demonstrated that blastocysts yielded more favourable postthaw survival rates with the use of vitrification for embryo cryopreservation (27,29). In the present study, although the viable embryos obtained after thawing were not subjected to embryo transfer, on the basis of the in vitro culture results it might be concluded that the transfer of the goat embryos in the blastocyst stage would yield better pregnancy rates.

It has been reported that, when the vitrification method is used for the cryopreservation of bovine, ovine, and caprine embryos, blastocysts and expanded blastocysts yield better results than morulae (27). According to the reports obtained so far, the higher postthaw survival rate of embryos in the blastocyst stage than in the

morula stage is related to the greater resistance of the cell membrane to osmotic and toxic stress after the formation of the embryonic blastocoel, along with increased active transport mechanism of cryoprotectants during blastocoel formation. Furthermore, differences in blastomere size could also affect the survival rate of embryos; morulae are more sensitive to osmotic stress during the removal of permeable cryoprotectants due to the fact that compact morula cells are larger than blastocyst cells.

The assessment of embryo quality is based on the morphological integrity of embryos. The morphological classification of embryos is done by grading embryo structure, cytoplasm density and colour, and the proportion of degenerated areas from 1 to 4 (1). It has been reported that, while embryos of Grade 1 (excellent) and Grade 2 (good) quality can be used for embryo transfer (ET) or can be frozen, those of Grade 3 (poor) quality can be frozen and used for ET only after being cultured (1,30). According to the results obtained for embryos classified as Grade 1 and 2 in the present study, on the basis of their morphological structure, it was determined that the postthaw survival rates at 24, 48, and 72 h were 78.6%, 46.4%, and 32.1%, respectively, for Grade 1 embryos, and 35.5%, 17.8%, and 17.8%, respectively, for Grade 2 embryos. These results showed that the postthaw survival rates of Grade 1 and Grade 2 embryos at 24 and 48 h differed significantly ($P < 0.05$). The postthaw in vitro survival rates of the embryos in the culture media significantly changed and decreased over time in relation to morphological integrity.

It was observed that the first 24 h was a very important period for embryo survival after the freeze-thawing of in vivo-derived goat embryos frozen with increasing doses of ethylene glycol and glycerol. The assessment of the postthaw in vitro survival rates of the goat embryos cryopreserved by vitrification at 24, 48, and 72 h in the culture media showed that the developmental stage and quality of the embryo during freezing were of major importance.

As a result, it was concluded that when the vitrification method is used for the cryopreservation of in vivo-derived Saanen goat embryos, on the basis of postthaw survival rates, embryos to be frozen should be selected on the basis of their developmental stage and quality, and embryos of Grade 1 (excellent/good) quality in the blastocyst stage should be preferred for freezing.

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