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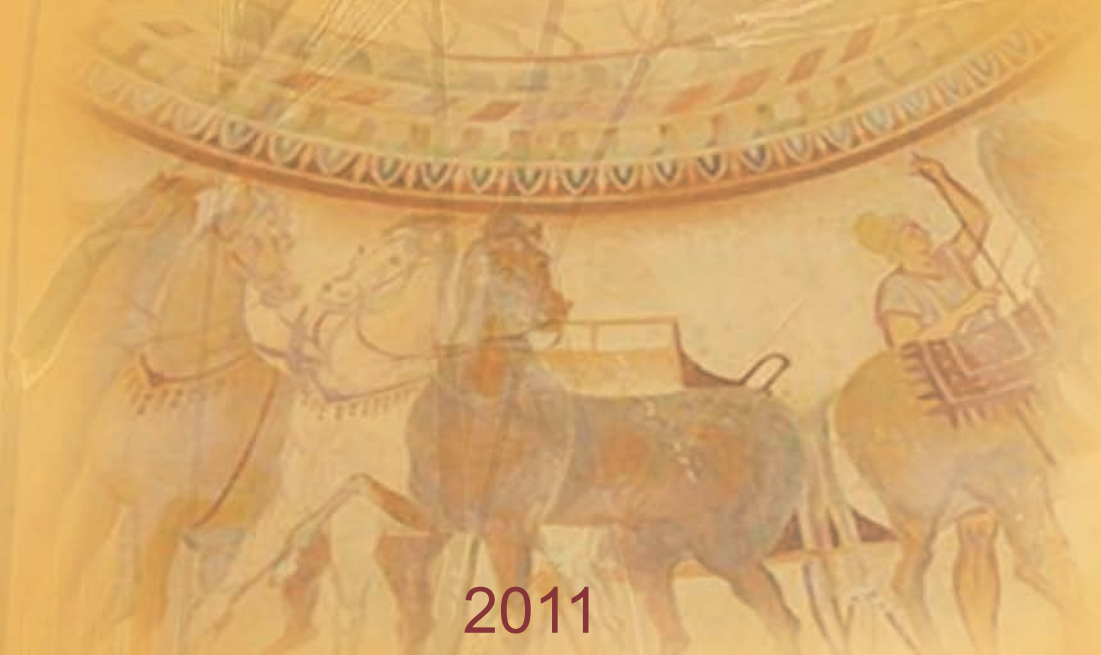

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Freezing of day 5 and 6 sheep and goat embryos of Greek breeds

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Abstract. *The aim of the present study was to investigate cryopreservation at day-5 and 6 of sheep embryos of Chios breed and goat embryos from indigenous Greek breed by the conventional one- or multi-step methods using glycerol and sucrose. Chios sheep and indigenous goats were super ovulated using FSH-P and embryos collected surgically five or six days after mating. Thirty two day-5 and 46 day-6 sheep embryos were frozen either by the one-step or multi-step method and stored in liquid nitrogen. Thirty five and 42 goat embryos were frozen by one-step or multi-step method, correspondingly. From these stock 48 sheep embryos and 56 goat embryos were thawed and transferred into 24 ewe and 28 goat recipients. None of the ewes that received one-step frozen embryos either of day-5 or day-6 lambed. From the recipients of multi-step sheep embryos 2 lambs /17%/ from day-5 and 6 lambs /50%/ from day-6 embryos were obtained. The goats that received one-step frozen embryos delivered 4 kids (25%) from day-5 and 4 kids (33%) from day-6 embryos and from multi-step goat embryos were obtained 4 kids (33%) from day-5 and 8 kids (50%) from day-6 embryos. It is concluded that day-6 multi-step frozen embryos, either of sheep or goats, produce more viable offspring after transfer in suitable recipients.*

Keywords: sheep, goat, embryos, freezing

Abbreviations: FSH – follicle stimulating hormone, MAP – medroxiprogesteronacetatum, PBS – phosphate-buffered saline, BSA – bovine serum albumin, PMSG – pregnant mare's serum gonadotropin, DMSO – dimethylsulphoxide

Introduction

Successful frozen preservation of ovine (Willadsen et al., 1976) and caprine embryos (Bulton and Moore, 1976) was reported by using the classical slow freezing and thawing procedures of Whittingham et al.(1972). Sheep embryos have proven more sensitive to the freezing procedures than mouse embryos and the first major used cryoprotectants were glycerol and sucrose. Glycerol acts by forming part of the solute, reduces the concentration of salt and controls the formation of intracellular and extracellular ice while sucrose, as a non-permeating cryoprotectant, acts in the extracellular environment and dehydrates the cell.

Freezing the embryos with the multi-step method involves freezing in two steps (room temperature until -3°C to -7°C and then until -40°C) and dilution of cryoprotectants, after rapid thawing, in consecutive steps where embryos remove from the straw (Schiewe et al., 1991). The other approach to the widespread use of cryopreserved sheep embryos on the farm is the one-step method that permits embryos to be diluted and transferred into a recipient without the need of laboratory equipment and specialists to examine and handle the thawed embryos before transfer (Le Gal, 1993).

The experiments reported here are the first attempt to assess the viability of day-5 and 6 sheep embryos of Chios breed and goat embryos of indigenous Greek breed, freezing with a one- or multi-step method. These experiments were performed as a part of an investigation project in order to evaluate and establish embryo transfer in Greek breeds of sheep and goats.

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Material and methods

Superovulation of donor

Ewes. The oestrous cycles of twenty Chios ewes were synchronized by the insertion of intravaginal sponges containing 60 mg MAP (Veramix, Upjohn) for 14 days. Two days before removal of the sponges the ewes were superovulated by an i.m. injection of 25mg FSH (FSH, Schering) in eight consecutive doses (4mg, 4mg, 4 mg, 3mg, 3mg, 3mg, 2mg, 2mg) with 12 hours' intervals.

Goats. Twenty four indigenous goats were synchronized by the insertion of intravaginal sponges containing 60 mg MAP (Veramix, Upjohn) for 17 days. Two days before removal of the sponges the ewes were superovulated by an i.m. injection of 21 mg FSH (FSH, Schering) in eight consecutive doses (4mg, 3mg, 3mg, 3mg, 2mg, 2mg, 2mg, 2mg) with 12 hours' intervals.

Twelve hours after removal of the sponges all females were tested for estrus with an approved male in the morning and afternoon, and they were mated with a fertile male of the same breed twice daily as long as they exhibited standing estrus.

Collection of embryos

The day-5 and 6 sheep and goat embryos (morulae, early blastocysts and blastocysts) were recovered from the donors under surgical collection procedures. A mid-ventral laparotomy was performed and each uterine horn was flushed by injection of Dulbecco's PBS (flushing medium) containing Ca and Mg and supplemented with 1% glucose, 3 % of BSA, 0,36 % sodium pyruvate, 0,20 % penicillin and 0,40 % streptomycin. The flushing

medium was injected through the fimbrial end of the oviduct and its collection was made from the end of the uterine horn, close to the body of the uterus. The embryos were pooled in PBS solution (culture medium), containing the same ingredients with flushing solution, except of BSA, which added in the proportion of 4%. The pooled embryos were classified according to their stage of development and quality (Lindner and Wright, 1983) and the classified as good or excellent were apportioned equally as appropriate for freezing.

Embryo freezing

The cryoprotectants used were glycerol and sucrose in solution in PBS-culture medium. The 5- or 6-day embryos were equilibrated at room temperature in the glycerol solution at increasing concentrations (0,5 M, 1,0 M and 1,5 M for 5-7 min in each) and were then placed into 0,25ml plastic insemination straws (IMV, L' Aigle).

Experiment 1: One-step freezing. Two embryos were drawn into each straw together with a small volume of 1.5 M glycerol, which was separated by two air bubbles from a 0, 5 M sucrose solution.

Experiment 2: Multi-step freezing. Two embryos were drawn into each straw together with a small volume of 1.5 M glycerol, which was separated by two air bubbles from a 1, 5 M glycerol solution, too. Each straw was identified and placed in the cooling chamber of a programmable ethylic alcohol-freezing unit (HAAKE, SK91). The cooling rate was 5°C/min from room temperature to -3°C. Seeding was induced manually at -3°C and the embryos were frozen to -32°C at the rate of -0, 3 °C/min. The straws were finally plunged into liquid nitrogen (-196°C) and stored for one year.

Embryo thawing

A thawing rate of approximately 1200°C/min was used by emerging the straws in a 37° water bath for 10 seconds.

Experiment 1: One-step freezing. The cryoprotectant was removed from the embryos by gentle agitation of the straws to mix the glycerol and sucrose media.

Experiment 2: Multi-step freezing. After thawing, the embryos were placed, for 5-7 min each, in three media at decreasing concentration of glycerol and increasing of sucrose (I: glycerol 1, 0 M + sucrose 0,3 M, II: glycerol 0,5 M + sucrose 0,4 M and III: sucrose 0,5 M).

The thawed embryos were placed in culture medium and examined under a stereoscope. Morphologically normal-looking embryos were transferred into recipient ewes.

Transfer of embryos

Thawed embryos were transferred also by laparotomy to 24 recipient ewes of Karagouniki breed and 28 recipient goats of an indigenous breed. The insertion of intravaginal sponges for 14 days and administration of 330 I.U. PMSG (Intergonan, INTER VET) synchronized the recipients at the time of sponge withdrawal. The exteriorized horn, which oviduct curried at least one corpus luteum, was punctured and two embryos per ewe were introduced in a small amount of medium through a plastic capillary pipette fixed to a 1ml syringe.

Pregnancy was determined by detection of estrus at 15^{th}-19^{th} day of estrous cycle for the ewes and 17^{th}-23^{rd} day for the goats and the pregnant recipients were allowed to proceed to term.

Statistical analysis

Data were tested using chi-square test. Results were considered as being significant at $p<0.05$.

Results

The embryo production of Chios ewes and indigenous goats is shown in Table 1. In Chios ewes from the 92 day-5 and 104 day-6 embryos recovered 32 and 46, correspondingly were frozen either by one-step or multi-step method and stored in liquid nitrogen. In goats from the 82 day-5 and 110 day-6 embryos recovered 35 and 42, correspondingly were frozen either by a one-step or multi-step method, too.

From these stock 48 sheep embryos and 56 goat embryos were thawed and transferred into 24 ewe and 28 goat recipients (Table 2). None of the ewes received one-step frozen embryos either of day-5 or day-6 was lambed. From the recipients of multi-step sheep embryos were obtained two lambs (17%) from day-5 and 6 lambs (50%) from day-6 embryos. The goats that received one-step frozen embryos delivered 4 kids (25%) from day-5 and 4 kids (33%) from day-6 embryos and from multi-step goat embryos were obtained 4 kids (33%) from day-5 and 8 kids (50%) from day-6 embryos.

Table 1. Embryo production of Chios ewes and indigenous goats superovulated by FSH

Embryo production	Day -5	Day – 6
Sheep		
Donor ewes	10	10
Corpora lutea	128	122
Recovered embryos	92	104
Embryos One-step frozen	16	23
Embryos Multi-step frozen	16	23
Goat		
Donor goats	12	12
Corpora lutea	122	128
Recovered embryos	82	110
Embryos One-step frozen	17	21
Embryos Multi-step frozen	18	21

Discussion

Embryo cryopreservation is an important adjunct to embryo transfer (ET). When an ET program is established for the first time in a breed it is necessary, among others, to estimate the efficiency of most common as well as up-to-date cryopreservation methods. The first success after transfer of a frozen-thawed embryo was obtained years ago (Willadsen et al., 1976; Bilton and Moore, 1976). Embryos were first stored in media containing glycerol, sucrose and dimethylsulphoxide (DMSO) as cryoprotectant. Survival rates of 30% to 60% have been reported after transfer of embryos frozen-thawed by conventional methods with a variety of cryoprotectants (Willadsen, 1977; Tervit and Goold, 1984; Heyman et al., 1987).

We found that day-5 and day-6 Chios sheep embryos were irreversibly damaged during the one-step freezing procedure (Rao et al., 1988; Cognie, 1999). The cause could be related to either inadequate intracellular equilibration prior to rapid cooling or to osmotic stress caused by incomplete removal of glycerol from the blastomeres during sucrose dilution (Schiewe et al., 1991). On the other hand, day-5 and day-6 Greek goat embryos are not so sensitive in one-step freezing and survive when they were subjected to dehydration with sucrose (Le Gal et al., 1993). The efficacy of

Table 2. Lambings and kiddings from frozen sheep and goat embryos transferred to recipient females

Embryos transferred	Sheep			
Freezing Method	One-step		Multi-steps	
Age of embryos	Day-5 (%)	Day-6 (%)	Day-5 (%)	Day-6 (%)
Recipient ewes	6	6	6	6
Embryos transferred	12	12	12	12
Ewes lambing			2	4
Lambs born			2(17)[a]	6(50)[b]
	Goat			
Freezing Method	One-step		Multi-steps	
Age of embryos	Day-5 (%)	Day-6 (%)	Day-5 (%)	Day-6 (%)
Recipient ewes	8	6	6	8
Embryos transferred	16	12	12	16
Ewes lambing	4	4	4	6
Lambs born	4(25)[a]	4(33)[b]	4(33)[c]	8(50)[d]

glycerol for sustaining Chios sheep embryo viability after cryopreservation was similar to that reported by Willadsen (1977) using DMSO. The sheep embryos posses the ability to equilibrate rapidly without experiencing blastomere lysis (Tervit and Goold, 1984). Also, Chios sheep and Greek goat embryo survival tended to be higher when glycerol was removed using sucrose in several steps of increasing concentrations (Lehn-Jensen et al., 1981; Lehn-Jensen, 1986). It was concluded that there is significant difference between age and survival post freezing as well as between embryos frozen by one-step or multi-step method. Day-6 embryos were more tolerant than day-5 embryos both in Chios sheep and Greek goat. The multi-step freezing method increased the number of offspring produced by either day-5 or day-6 embryos. Day-6 Chios sheep and Greek goat embryos frozen by a multi-step procedure resulted in high embryo survival and pregnancy rates when transferred to recipients.

In this paper we worked with classical freezing methods as a first approach to freezing Chios sheep and indigenous Greek goat embryos. Our next approach will be the use of ethylene-glucose which has emerged as a superior cryoprotectant (Thibier and Guerin, 2000) with higher survival rates approaching those achieved with fresh embryos and with the possibility to transfer directly the embryo after thawing. Also, sheep and goat embryos are able to survive vitrification procedures and with further research this method may provide an economical alternative to the current freezing methods requiring gradual dehydration of embryonic cells.

Conclusion

There is significant difference between age and survival post freezing as well as between embryos frozen by one-step or multi-step method. Day-6 embryos were more tolerant than day-5 embryos both in Chios sheep and Greek goat. The multi-step freezing method increased the number of offspring produced by either day-5 or day-6 embryos. Day-6 Chios sheep and Greek goat embryos frozen by multi-steps procedure resulted in high embryo survival and pregnancy rates when transferred to recipients.

References

Bilton RJ and Moore NW, 1976. In vitro culture storage and transfer of goat embryos. Australian Journal Biological Science, 32,101-107.

Cognie Y, 1999. State of the art in sheep-goat embryo transfer. Theriogenology, 51,105-116.

Heyman Y, Gamier V and Cognie Y, 1987. Transfer of frozen-thawed embryos in sheep. Veterinary Record, 120,83-85.

Le Gal F, Baril G, Vallet JC and Leboeuf B, 1993. In vivo and in vitro survival of goat frozen embryos with ethylene glycol or glycerol. Theriogenology, 40, 771-777.

Lehn-Jensen H, Greve T and Peresnava A, 1981. Two step freezing of cow embryos in 1.4 M glycerol. Theriogenology, 19,263-277.

Lehn-Jensen H, 1986. Cryopreservation of bovine embryos. The Royal Veterinary and Agricultural University Institute for Animal Reproduction. Ed. A/S C.Fr. Mortensen, pp,183.

Lindner GM and Wright RW, 1983. Bovine embryo morphology and evaluation. Theriogenology, 20, 407-416.

Rao VH, Sarmah BC, Agrawal KP, Ansari MR and Bhattacharya NK, 1988. Survival of goats embryos frozen and thawed rapidly. Animal Reproduction Science., 16, 261-264.

Schiewe MC, Rail WF, Stuart LD and Wildt DE, 1991. Analysis of cryoprotectant cooling rate and in situ dilution using conventional freezing or vitrification for cryopreserving sheep embryos. Theriogenology, 36, 279-293.

Tervit HR and Goold PG, 1984. Deep-freezing sheep embryos. Theriogenology, 21, 268.

Thibier M and Guerin B, 2000. Embryo transfer in small ruminants: the method of choice for health control in germplasm exchanges. Livestock Production Science, 62, 253-270.

Whittingham DG, Leibo SP and Mazur P, 1972. Survival of mouse embryos frozen to -196 °C. Science, 178, 411-414.

Willadsen SM, Polge C, Rowson LE A and Moor RM, 1976. Deep-freezing of sheep embryos. Journal of reproduction and Fertility, 46,151-154.

Willadsen S M, 1977. Factors affecting the survival of sheep embryos during deep freezing and thawing. In: "The Freezing of mammalian Embryos", Ciba Foundation Symposium, 52,175-202.

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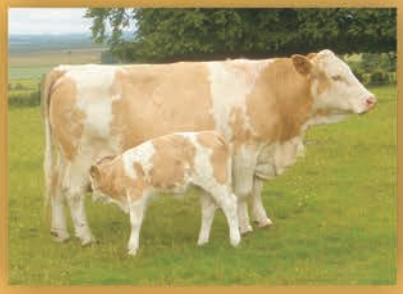

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