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CONSERVATION OF ANCIENT BREED SMALL RUMINANTS AS FROZEN EMBRYOS

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Summary

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A total of 69 sheep and goat embryos at the stage morula or blastocyst produced *in vivo* from endangered native Bulgarian breeds were recovered, frozen and stored in a cryobank with liquid nitrogen at –196°C. The methods for production, recovery and deep-freezing of the preimplanted embryos are described. The specific hormonal stimulation and possibilities for freezing biotechnology as well as a long-term storage in animal biology science and stock-breeding are discussed. It is well demonstrated that embryo freezing is an approach for conservation of the animal genotype at its current status for a prolonged period.

Key words: conservation, embryo, freezing, goat, sheep

INTRODUCTION

Numerous biological species are under immediate threat of extinction at the beginning of the present century. Therefore besides the preservation of natural genetic fund in safari parks, zoos and stock-breeding farms, the conservation of germ cells and embryos of rare and disappearing animals with specific genetic qualities is necessary for preserving of the genetic resources of the national fauna. The embryo contains the full genetic complement predetermined by the mother and the father. It is a more reliable object for cryopreservation and reproduction of the parents as compared to spermatozoa and oocytes. Deep-freezing of mammalian embryos is a biotechnological approach for the preservation of the hereditary genetic features of many animal species. Its major goal is the reproduction of individuals, breeds and species after storage and embryo transfer in appropriate recipient(s). The first successful cryopreservation of mouse embryos has been realized in England about three decades ago by Wittingham (1971). With time, due to the development of the cryobiological knowledge and the improvement of cryogenic techniques, the embryos of several farm animal species have also been successfully frozen, stored in cryoembryobanks and additionally transferred (Willmut & Rowson, 1973; Bank & Maurer, 1974; Bilton & Moore, 1976; Willadsen *et al.,* 1976; Dobrinsky, 2002).

In Bulgaria, Vlahov *et al.* (1981) and Sapundzhiev *et al.* (1991) have initiated investigations on embryo freezing, following transfer of frozen-thawed embryos, resulting in the birth of lambs and calves.

The present article comprises the results of production, freezing and storage of early preimplanted embryos at morula and blastocyst stages, recovered from

small domestic ruminant donors. The animals of these native breeds differ significantly with respect to certain genetic, morphological and functional features as well as productive performance when compared to animals raised at other regions of the country. Due to socio-economical changes in Bulgaria, the animal population has been strongly reduced and the indigenous breeds are particularly in danger of extinction, being reared in few private and cooperative farms.

MATERIALS AND METHODS

Sheep from the Shumen Copper-red (n=10), Northeastern Bulgarian (n=8), Karakachan (n=9) breeds and goats of the indigenous Bulgarian breed (n=8) were monitored and included in the donor groups. The donors were located at various regions of the country and the trials were done during the three-year period of the investigated project.

The oestrus of small ruminants was synchronized by using progestagen impregnated sponges (40 mg Chronogest, Intervet). The ewes and goats were stimulated for superovulation with 15 mg FSH (Burns, Biotec) given in 4 doses (5, 5, 3 and 2 mg) at 12-hour intervals, before the sponges were removed on the 14th day or treated with 500 U.I. PMSG (Folligon, Intervet) injected on the same day. Sheep were artificially inseminated twice at a 12-hour interval by frozen semen on the day of the heat, whereas goats were inseminated artificially with fresh semen. The embryos were collected via midventral laparotomy on the 6th day after insemination.

Collected oocytes and embryos were evaluated morphologically under a light stereo microscope at magnification 24–96×. They were divided in four groups – unfertilized oocytes (UO), degenerated embryos (DGE), morula (M) and blastocyst (Bl). Only intact morulae and blastocysts were included in the freezing programme. Half of the intact embryos of each group were randomly determined to be frozen with either glycerol or ethylene glycol. The freezing medium was prepared by addition of 20% decomplemented foetal calf serum to the basic Dulbecco's medium. The cryoprotectant (1.37M glycerol or 1.5M ethylene glycol) was added on three increasing steps for 10 min each. The freezing procedure used in the trials was a modification of the so-called "two-step method" (Whittingham, 1971):

1. The embryos were equilibrated at a temperature of 20°C in three consecutive solutions with increasing concentrations of cryoprotectants glycerol (0.45; 0.9 and 1.37 M) or ethylene glycol (0.5; 1 and 1.5 M) respectively, for 10 min in each solution.

2. The embryos were aspirated one by one in 0.5 mL straws (IMV, Cassou, France) that, after plugging and indication, were set in the automatic programmable biofreezer Minicool AS - 25 (L'Air liquide, France).

3. The samples were cooled from room temperature 20°C to −7°C at a rate of 1°C/min, then equilibrated for 5 min, seeded and equilibrated again for another 5 min.

4. Finally, the embryos were frozen at a rate of 0.3°C/min to temperature −35°C and then the straws were directly transferred into liquid nitrogen at −196°C for a long-term storage.

RESULTS

The summarized results of the trials with treated donors, recovered oocytes and

Table 1. Recovered and frozen embryos from small ruminant donors.

Donor breed	Recovered oocytes and embryos				
	unfertilized oocytes	degenerated embryos	morulae	blastocysts	frozen embryos
Shumen Copper-red sheep (n=10)	7	2	20	-	20
Northeastern Bulgarian sheep (n=8)	30	11	3	7	10
Karakachan sheep (n=9)	50	7	13	-	13
Indigenous Bulgarian goat breed (n=8)	16	4	24	2	26

frozen embryos are shown in Table 1.

From 27 donor sheep, 107 unusable unfertilized oocytes (UFO) and degenerated embryos (DGE) and 20 UFO and DGO from 8 donor goats were recovered, respectively. A total of 36 morulae (M) and 7 blastocysts (Bl) from sheep embryos and respectively 24 M and 2 Bl from goat embryos were chosen for freezing. Altogether 69 frozen embryos from small ruminant donors were stored in liquid nitrogen container at a temperature –196°C as cryoembryobank.

The observed osmotic changes in the blastomers of the embryos subjected to freezing were a functional test of their viability during the equilibration in the cryoprotective medium. The dehydration proceeded with the characteristic temporary shrinkage of the blastomers, reduction of the cell volume and increase of the space between blastomers and zona pellucida. Signs of lysis or hypertension were not observed. At present, the frozen embryos are stored in liquid nitrogen container.

DISCUSSION

In the group of small ruminants there was a variation of the percentage UFO and DGE in ewes and goats. The higher percentage of the UFO and DGE in the ewes (3.96% per donor) was probably due to using frozen semen for artificial insemination. The smaller percentage of the UFO and DGE in the goats (2.5% per donor) could be attributed to the use of fresh semen. Nevertheless, almost ⅔ of the recovered ova and embryos were excluded from the freezing programme as unsuitable. This was probably due to the conservative reproductive system of the indigenous farm animals after hormonal treatment. Embryos in the stages of morula or blastocyst with normal appearance and characteristic structure for the stage of development were chosen for freezing. The evaluation criteria depend on the accumulated experience and technical equipment, despite the attempts for unification (Cognie *et al.,* 2003). Additional evaluation of the survival is needed in the future by *in vivo* or *in vitro* tests.

The embryos were equilibrated in cryoprotective medium at three steps for 10 min each, since experiments with labeled carbon have shown that the optimal time for accomplishment of the osmotic processes of dehydration and penetration of the cryoprotectant was 30 min (Jackowski *et al.,* 1980). Glycerol is commonly used for animal embryo freezing because of its low toxicity and its property to form small crystallization nuclei of intracellular water which facilitates the storage at subzero temperatures (Dobrinsky, 2002). There are also reports that ethylene glycol is widely used for freezing sheep embryos (Cocero *et al* 1996), but our experience indicates less reliable results with this cryoprotectant in other experiments (Sapundzhiev *et al.,* 1991). The sheep and goat embryos appeared more viable after the freezing-thawing procedures and following test *in vitro* when glycerol was used.

At present, a total of 69 sheep and goat deep-frozen embryos are stored in a cryobank at –196°C more than 10 years. The realization of embryo freezing programmes can contribute to the preservation of the genuine genotype and phenotype of endangered indigenous national breeds and species. It is well known that some photophysical reactions can occur into deep-frozen cells such as the formation of free radicals and portions of macromolecules. Furthermore there is no evidence that germ cells storage into liquid nitrogen results in the accumulation of chromosomal or genetic changes (Massip, 2001). The storage of the inherited qualities of the genome of biological species in its present status is of great significance since with time, a genetic drift is often manifested which provokes changes in breeds and species and is a source of mutability and evolutionary development. Moreover, embryo cryopreservation is considered as a form of biological protection, which in future might contribute to the maintenance of the genetic stability and diversity in animal kingdom (Dobrinsky, 2002). The genotype and phenotype can be easily restored and the animals will at the disposal of breeders after thawing and successful embryo transfer in appropriate recipients (Cognie *et al.,* 2003).

On the other hand, there is information that methods for vitrification can be successfully applied to cryopreserve embryos from various animal species (Massip, 2001). It seems that future efforts should to be concentrated on this direction although there are real evidences of oocyte and embryo sensitivity to the osmotic and low-temperature stress of the vitrification procedure.

Cryoembryobanking, conventional or experimental, has a practical application in both animal husbandry and fulfillment of contemporary ecological programmes aiming at preservation of endangered animals. It is also of a great scientific significance for the basic reproductive biology and genetics.

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