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Comparative Analysis of Two Embryo Freezing Methods

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Two rates of methods are mainly conducted to establish the influence of the deep embryo freezing protocol on the post-thawing viability during the embryobiotechnology procedure.

The aim of the present study is to compare reliability of both so call conventional slow-rate freezing and vitrification high-speed method, respectively for preservation the viability of animal preimplanted embryos.

Using slow-rate freezing live offspring were produced from Romanovska sheep frozen-thawed embryos subsequently transferred to synchronized different breed recipients. The viability rate ranged between 40% and 57% when 1.37 M glycerol was used in two experiments. The beef cattle embryos were treated just the same speed protocol and their viability was 40% and 25% when 1.37 M glycerol and 1.5 M ethyleneglycol were used respectively, when new-born calves were obtained. In comparison vitrified mouse embryos survive the freezing with 6.85 M glycerol cryoprotectant and viability was saved up to 44% after in vitro culture.

Key words: freezing, embryo, embryobiotechnology.

Introduction

Embryo freezing is a cryobiology method achieving arrest of the biological processes in their blastomeres. Following manipulations of thawing and dilution of the cryoprotectant restore the embryo viability and give as an attractive tool of controlling animal reproduction. After the first successful approach of freezing-thawing mouse embryos [12], various mammalian embryo cryopreservation procedures have been developed and gave acceptable in vitro or in vivo results abroad and also in our country [9]. These procedures can be divided into two main types – conventional slow-rate freezing and vitrification. Various cryoprotectants were used for freezing of animal embryos and their influence on viability rate was examined after thawing in proper conditions – in vitro culture or embryo transfer.

Conventional freezing is the method in which the cryoprotectant concentration reaches to 10% and good post-thawing viability rate has been achieved. Many kinds of cryoprotectants such glycerol, DMSO, ethyleneglycol and propyleneglycol were used. The disadvantage of this method is the necessity of special costly equipment which real-

ized the computerized program for a long time period (2-3 h) of freezing. The method was elaborated earlier and has wider application in practice.

Vitrification method has the advantages of being much quicker (a couple of min) and cheaper. Otherwise, this method is still experimental and higher up to 50% concentration a mixture of various cryoprotectants has expressive toxicity.

The aim of the present investigation was to compare viability rate of animal preimplanted embryos after freezing, thawing and subsequent embryobiotechnology of so called slow-rate procedure and vitrification method respectively.

Materials and Methods

Animal donors and embryo recovery

Sheep. Two subsequent experiments A and B were provided with 8 Romanoska sheep respectively 5 and 3. Superovulation of the donors was induced by intramuscular injection of 1000 IU PMSG (Pregmagon, Germed, Germany) on day 10 of the sexual cycle (estrus = day 0). The donors were treated 48 h later with analogue of PGF2α – 125 μg Cloprostenol (Oestrophan, Spofa, Czech Republic). The sheep were artificially inseminated twice in 12-hour interval with fresh semen when clinical signs of estrus were observed. A total of 28 embryos respectively 19 in trial A and 9 in trial B were recovered on day 6 of gestation by midventral laparotomy and flushing uterine horns. The obtained embryos were in blastocyst stage in experiment A and morula and early blastocyst in experiment B. Manipulating medium (MM) was phosphate buffered saline (PBS) solution – Dulbecco's medium supplemented with 20% decomplemented fetal calf serum. The freezing medium (FM) was prepared by addition of 1.4 M glycerol to MM on three steps for 30 min. The embryos individually were placed in 0.5 ml straws (IMV, Cassue, France) containing FM. The experiment A with frozen-thawed sheep embryos was done in Bulgaria. The experiment B was international and embryos in frozen condition were ship to Poland.

Cattle. A total of 17 beef cattle (breed Hereford – n=12, Salers – n=3, and Aberdeen-Angus – n=2) were hormonally stimulated with 3500 IU Pregmagon (Dessau, Germany) intramuscular injection on the day 10 or 11 of the estrus cycle. The cows exhibited heat after 2 days were inseminated twice with frozen-thawed sperm of a relevant breed bull at a 12-hours interval. The embryos at morula and blastocyst stage were recovered on the day 7 after insemination by a transcervical flushing of the uterine horns using catheters type Foley (Willy Rush, Germany) and solution MM. Altogether 81 embryos only intact morulae and blastocysts were included in the freezing programme. The FM was two modifications. The first one was compounded of cryoprotectant 1.4 M glycerol for 50 embryos. The second was consisted of 1.5 M ethyleneglycol for 31 embryos. The final concentration was obtained by addition on three increasing steps for 30 min. The embryos were separately placed in 0.5 ml straws (IMV, Cassue, France) previously filled with corresponding FM. All the embryos were ship to the Russia in frozen condition and embryo transfers to 27 Holstein-Friesian recipients altogether were accomplished.

Mice. Hormonal stimulation was induced to 7 female BALBc fertile mice by intraperitoneal injections of 5 IU PMSG (Gestyl, Organon, Holand) and 5 IU hCG (Pregnyl, Organon, Holand) given 48 h apart and couple was made. The presence of the vaginal plug on the next day was evidence for mating. The embryos were collected 72 h after that by forceps of the oviduct at 8-16 blastomeres stage. The mice were in general narcosis and the ethical principles and legal requirements for the welfare with the animals

were kept during the embryo recovering using MM. The freezing medium (FM) was prepared by addition of 6.85 M glycerol to MM. The embryos in a group were placed in 0.5 ml straws (IMV, Cassue, France) with content of FM.

Freezing and thawing procedure

The sheep and cattle embryos were frozen by conventional slow-rate method in program biofreezer Minicool AS 25 (L'Air Liquide, France) according to the so called "two-step method":

1. Cooling of the straws from temperature 20°C to –7°C with rate 1°C/min.
2. Equilibration for 5 min, seeding of the crystals and subsequent 5 min equilibration.
3. Freezing to temperature –30°C with a rate 0.3°C/min.
4. Transfer of the straws directly to container with liquid nitrogen to temperature –196°C.

Thawing was accomplished by immersion of the straws in water bath at temperature 37°C. Cryoprotectant dilution was done in accordance of the cryoprotectant. Glycerol was diluted in four steps by transfer the embryos in equilibrated at room temperature solution with decreasing concentration 1 M, 0.7 M, 0.3 M for 5 min each step and finally in culture medium. Ethyleneglycol was diluted by placing the embryos in fresh freezing medium containing 0.25 M sucrose for 10 min at room temperature and than placed in manipulating medium.

The mouse embryos were frozen by direct transfer of the straws in liquid nitrogen at -196°C. They were thawed by immersing of the straws in water bath at 38°C for 1 min. For cryoprotectant diluted medium (DM) 0.35 M sucrose was added to FM and the embryos remained for 10 min.

Biotechnological assessment of the viability.

Sheep embryos were surgically transferred to 17 recipients from the breeds Bulgarian Cigai in experiment A and Polish plane crossbreed in experiment B respectively 10 and 7 in number.

Cattle embryos were nonsurgically transferred to synchronized 27 Holstein-Friesian breed recipients in Russia.

Pregnancy and parturition in all trials were registered in comprehensive protocol.

Mouse embryos were in vitro cultured in 5% CO_2 incubator at 38°C in tissue culture medium. Development to the stage expanded or hatched blastocyst was sign for embryo viability.

Results and Discussion

The viability of sheep embryos at morula and blastocyst stage frozen conventionally with 1.4 M glycerol was evaluated in two experiments summarized in Table 1. In experiment A from 19 frozen embryos 10 were chosen for transfer. A total of 9 frozen embryos were excluded of the experiment after thawing and dilution of the cryoprotectant for presence of morphologic discrepancy. The viability of frozen embryos was evaluated up to 40% and 4 offspring were obtained from 10 recipients. In experiment B viability of 9 frozen embryos reached to 57% and 4 newborn lambs were registered from 7 transferred embryos after thawing and dilution of the cryoprotectant to 7 recipients. Only 2 embryos were evaluated as unsuitable for embryo transfer because of degeneration and lysis after dilution of the cryoprotectant.

Our results with sheep embryos demonstrated that conventional slow-rate freezing method using 1.4 M glycerol is proper for preimplanted stage of development. Our originally experience has shown that embryo viability depended upon the stage of embryo development, rates of cooling and thawing or choice of cryoprotectant. Some authors were used DMSO or ethyleneglycol for freezing sheep embryos but the success is variable [1, 2].

The viability of cattle embryos was estimated after slow-rate freezing method comparing two kinds of cryoprotectants – glycerol and ethyleneglycol and the result is shown in Table 2. From 50 frozen with 1.4 M glycerol embryos belonged to Hereford breed cattle after thawing and stepwise dilution of the cryoprotectant 24 (48%) were evaluated as proper for embryo transfer to 15 recipients. As a result 7 pregnant cows were registered by rectal palpation on the third month of gestation. The viability achieved for this group was 6 (40%) newborn calves, respectively male and female equally. The cow suffer abortion was pregnant with twins. In the group of 31 cattle embryos frozen with 1.5 M ehtyleneglycol 5 were Hereford 17 – Salers and 9 – Aberdeen-Angus. After thawing-dilution procedure 12 (39%) embryos were eliminated of the trials as unsuitable for embryo transfer. A total of 19 (61%) embryos were transferred to 12 recipients. The viability rate was registered as 3 (25%) offspring female calves 1 of them Hereford and 2 Salers. As a final result 9 newborn calves were registered. Out of them 7 calves were Hereford breed and 2 – Salers breed. No Aberdeen-Angus breed was delivered of transferred embryos.

The presence of a cryoprotectant is required to avoid damage to embryos during freezing and thawing. Since the presence of a cryoprotectant significantly increases the medium's osmolality which could damage the embryos, these substances have been mostly added in a step-wise manner [4, 7]. Our results show that different cryoprotectants preserved embryo survival in accordance of their permeability, respectively 1.4 M glycerol up to 48% and 1.5 M ethyleneglycol up to 61%. They are comparable to the obtained pregnancy rate following the transfer of frozen–thawed embryos of the other authors using similar approach [5, 6]. On the other hand the subsequent effectiveness is discussible because the viability vas saved up to 40% when glycerol was used. Contrary to expectation the viability was decreased up to 25% for embryos frozen with ethyleneglycol. The distinctions may probably due to different approach of cryoprotectant dilution and expressed toxicity.

The results of vitrified than thawed and in vitro cultured mouse embryos are summarized on Table 3. The experimental group of 152 embryos in early morula stage of development with expanded blastomeres and intact zona pellucida were assessed morphology normal and divided in 2 groups. After vitrification and following thawing procedure of 138 embryos the blastomeres were contractive and zona pellucida was intact at 113 (82%) embryos and they were morphologically evaluated as survived. Dilution of the cryoprotectant indicates initial shrinkage and subsequent swelling to the normal isotonic volume. A signs of zona pellucida hardening was also observed which reduced permeability of the cryoprotectant. Nevertheless the procedure decreased the survival rate and 96 (70%) embryos showed rehydration of the blastomeres. The results of in vitro embryo culture on 24 h demonstrated viability rate reached to blastocyst stage for 78 (56%) embryos and comparable saved viability on 48 h of cultivation up to stage hatched blastocyst of about 61 (44%) embryos respectively. A control group of 14 embryos were only cultured without applying on the freezing program. Their viability was 86% on 24 h of cultivation at blastocyst stage and 71% on 48 h at expanded or hatched blastocyst respectively.

During the vitrification of mouse embryos the movement of the cell volume and decreasing of the space between blastomeres and zona pellucida were additional indica-

Table 1. Survival and in vivo viability of conventionally frozen sheep embryos using different recipients

Trial	Donors (n)	Frozen embryos	Transferred embryos	Recipients (n)	Newborn lambs (%)
A	Romanovska (5)	19	10	Cigai (10)	4 (40)
B	Romanovska (3)	9	7	Polish plane (7)	4 (57)

Table 2. Survival and in vivo viability of conventionally frozen beef cattle embryos using different cryoprotectants

Cryoprotectant	Donors (n)	Frozen embryos	Transferred embryos	Recipients	Pregnant on 3rd month	Newborn calves (%)
Glycerol	Hereford 10	50	24	15	7	6 (40)
Ethyleneglycol						(25)
	Hereford 2	5	5	5	2	2
	Salers 3	17	10	5	1	1
	Aberdeen-Angus 2	9	4	2	-	-

Table 3. Survival and in vitro viability of vitrified preimlanted mouse embryos

Methods	Treated embryos	Embryo survival rate		Embryo viability rate	
		normal embryos after thawing (%)	normal embryos after dilution (%)	after 24 h culture *in vitro* (%)	after 48 h culture *in vitro* (%)
Vitrification	138	113 (82)	96 (70)	78 (56)	61 (44)
Nonvitrified	14	-	-	12 (86)	10 (71)

tions for embryo survival. Symptoms of lysis and hypertension were signs for embryo morphology destroy. The dilution of the cryoprotectant by one-step procedure using 0.35 M sucrose is commonly used in other vitrification protocols [8, 10]. Our results also demonstrated that achievement of high viability rate dependent on every method's step. It is also showed that development of mouse embryos were preserved after vitrification procedure like gene expression in mouse pronuclear zygotes [3]. In comparison similar results have been reported when the same protocol was applied to mouse and bovine early preimplanted embryos or using mixture of ethyleneglycol and propanediol vitrification solution [7, 11].

Conclusion

The present study demonstrated that slow-rate freezing and high-speed vitrification methods can be routinely used for animal embryos cryopreservation and their viability is saved ranged between 25-57 % *in vivo* and 44-56 % *in vitro,* respectively.

References

1. Cocero, M. J., R. Procureur, J. De La Fuente, D. Chupin. Glycerol or ethylenglycol for cryoprotection of deep-frozen ewe embryos. – Theriogenology, **29,1,** 1998, 238.
2. Cognie, Y., G. Baril, N. Poulin. Current status of embryo technologies in sheep and goat. – Theriogenology, **59,** 2003, 171-188.
3. Dhali, A., V. M. Anchamparuthy, S. P. Butler, R. E. Pearson, I. K. Mullarky, F. C. Gwazdauskas. Gene expression and development of mouse zygotes following droplet vitrification. – Theriogenology, **68,** 2007, 1292-1298.
4. Galli, C,, R. Duchi, G. Crotti, P. Turini, N. Ponderato, S. Colleoni, I. Lagutina, G. Lazzari. Bovine embryo technologies. – Theriogenology, **59,** 2003, 599-616.
5. Jousan, F. D., M. D. Utt, S. S. Whitman, R. H. Hinshaw, W. E. Beal. Effects of varying the holding temperature and interval from collection to freezing on post-thaw development of bovine embryos in vitro. – Theriogenology, **61,** 2004, 1193-1201.
6. Martinez, A. G., G. M. Brogliatti, A. Valcarcel, M. A. de las Heras. Pregnancy rates after transfer of frozen bovine embryos: a field trial. – Theriogenology, **58,** 2003, 963-972.
7. Massip, A. Cryopreservation of embryos of farm animals. – Reprod. Domest. Anim., **36,** 2001, 49-55.
8. Rall, W. F. Cryopreservation of oocytes and embryos methods and applications. – Anim. Reprod. Sci., **28,** 1992, 237-245.
9. Sapundzhiev, E. Freezing of preimplanted mouse embryos by vitrification. – Acta Morphologica et Antropologica, **13,** 2008, 116–120.
10. Scheffen, B., P. Van Der Swalmen, A. Massip. A simple and efficient procedure for preservation of mouse embryos by vitrification. – Cryo-Letters, **7,** 1986, 260-269.
11. Vajta, G. Vitrification of the oocytes and embryos of domestic animals. – Animal. Reprod. Sci., **60-61,** 2000, 357-364.
12. Whittingham, D. G. Survival of mouse embryos after freezing and thawing. – Nature, **233,** 1971, 125-126.