Survival of rabbit embryos after rapid freezing and thawing[1]

V.H. Rao, I.V. Mongha, M.R. Ansari and N.K. Bhattacharyya

Indian Veterinary Research Institute, Izatnagar-243 122 (India), 25 April 1983

Summary. Frozen storage of rabbit embryos at the 16-cell stage in 2.0 M dimethylsulfoxide (DMSO) in phosphate-buffered saline (PBS) was achieved by a 2-step procedure. After storage for 10 days at −196 °C they were revived by rapidly thawing at 500 °C/min. On transfer of these embryos to pseudopregnant foster mothers, 50% survived to term. The difference in in vivo survival between frozen-thawed and frozen-thawed-cultured embryos was not significant.

Since the discovery of the possibility of preserving mouse embryos by slow freezing and thawing[2], successful preservation of embryos at 196 °C has been achieved in rat[3], rabbit[4–7], cow[8–9], sheep[10] and goat[11]. However, slow freezing – slow thawing technology is inconveniently time consuming. Recently, 8-cell mouse embryos were successfully frozen by a 2-step method[12] and the embryos survived after thawing at a rate of 500 °C/min. Morulae of the mouse were also successfully frozen preserved by a similar method of cooling and the embryos survived thawing at 300 °C/min[13]. We report here for the first time a similar method for cryopreservation of 16-cell rabbit embryos which yielded high post-thaw survival in vivo.

Materials and methods. Animals. Individually-caged albino does and bucks bred for many generation at the Division of Laboratory Animal Research of the Institute were used in 5 sets of experiments. Donor does were superovulated[14] and mated to fertile bucks. Recipient does were mated to vasectomized bucks to induce pseudopregnancy.

Embryos. 16-cell embryos were collected 48 h after mating[15] and embryos with symmetrically cleaved blastomeres were chosen for preservation. All the embryos were washed twice with phosphate buffered saline (PBS)[16] without any antibiotic.

Freezing. 8–10 embryos were directly transferred in 0.20 ml of prechilled (4 °C) 2.0 M DMSO in PBS to 5 ml capacity screw-cap glass tubes. The tubes were allowed to stand for 10 min in an ice-bath. They were then transferred to an ethanol-ice bath (−10 °C) where the samples were seeded at about −6 °C and kept for 10 min. Then the tubes were held for 20 min. in liquid nitrogen (LN_2) vapours at −20 °C (by adjusting the distance of the tubes from the surface of the LN_2). The tubes were finally plunged into LN_2 and stored for 10 days.

Thawing. A thawing rate of approximately 500 °C/min was used by gently agitating the tubes in a hot water bath at 40 °C[12]. The thawing rate was calculated from the time taken for the temperature of the samples to rise from −60 °C to −10 °C[12]. As the tubes reached 20 °C, 0.1 ml of 1.0 M sucrose in PBS was added twice at 10-min intervals and the embryos were promptly recovered, washed with PBS, and examined under a microscope.

Transfer. Morphologically normal embryos were transferred (8–10/doe) into the Fallopian tubes of suitably timed pseudopregnant recipient does[15]. Remaining frozen-thawed embryos were cultured in vitro following a method standardized in our laboratory[17].

Results. Out of 148 embryos frozen-thawed in 5 experiments (table) 93% were recovered in morphologically excellent condition. 70 of these embryos were transferred immediately after thawing, which resulted in the birth of 37 rabbits, 29–31 days after transfer (table). On culture of 46 frozen-thawed embryos for 24 h all the embryos reached the morula/early blastocyst stage. Transfer of 28 of these cultured embryos resulted in 12 live young (table). The difference between in vivo survival rates of a) frozen-thawed and b) frozen-thawed-cultured embryos was not significant.

Discussion. The survival rate of rabbit embryos with a technique involving a slow rate of freezing-thawing was reported to be low[4–6] except in 1 instance[7] in which the number of embryos transplanted was small. Our results for survival (50%) are indeed higher than those reported earlier. There are indications that the 16-cell rabbit embryo is more tolerant than other cell stages[7]. Our results support this view, as 16-cell rabbit embryos survived the 2-step freezing and rapid thawing method well. Kasai et al.[13] observed better results with a thawing rate of 300 °C/min for mouse morulae, in comparison with 25 °C/min. In our experiment with rabbit embryos, we preferred to use a faster rate of thawing (500 °C/min) in consideration of the fact that the rabbit embryo blastomeres are much larger than those of the mouse at any cell stage. Since mammalian embryos differ in cryoprofiles (freeze-thaw requirements) depending on cell stages and species[18], it is difficult to assess the extent of improvement due to modification of the thawing rate. But it is certain from our results that the post-thawing survival of rabbit embryos, based on an in vitro culture parameter, was superior to that of mouse morulae[12]. Work is at present under way, in our laboratory, to determine whether the modified 2-step-method of freezing and faster thawing is equally suitable for other mammalian species. A developmental delay has been observed in mouse embryos after freezing-thawing[18]. The survival of such embryos was found to be improved by subjecting them to a period of in vitro culture prior to transfer[18]. In rabbits the differences in survival rate of frozen-thawed and frozen-thawed-cultured embryos were less pronounced (50% vs 43%). Frozen preservation of mammalian embryos has many applications in diverse fields[19]. Laboratory animals can be maintained as frozen embryos to conserve space and reduce costs. Further, important strains and mutants can be protected against loss through disease, accident or genetic drift. In animal husbandry, wide dissemination of female germ plasm can be achieved. International transport of frozen embryos is less risky and economical in comparison to transportation of animals. In fundamental research, embryos are being used as model systems to investigate cryobiological phenomena. Fro-

Frozen preservation of rabbit embryos

Exp. number	Embryos frozen	Embryos recovered (%)	Embryos transferred*	No. of bunnies born (%)	Embryos cultured**	Embryos transferred***	No. of bunnies born (%)
I	24	22 (92%)	10	4 (40%)	7	–	–
II	40	35 (87.5%)	20	11 (55%)	–	–	–
III	22	22 (100%)	20	11 (55%)	–	–	–
IV	31	29 (94%)	10	7 (70%)	19	18	8 (44%)
V	31	30 (97%)	10	4 (40%)	20	10	4 (40%)
Total	148	138 (93%)	70	37 (52%)	46	28	12 (43%)

*Transferred immediately after thawing; **Frozen-thawed and then cultured; ***Frozen-thawed-cultured and transferred.

zen preservation of embryos also permits stockpiling of embryos for biochemical analysis. The present method of rapid freezing and thawing of rabbit embryos is less tedious than those reported hitherto, requires no specialized equipment, and permits higher survival rates. Thus it should be more useful in practice.

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Endurance training and antioxidants of lung[1]

A. Salminen, H. Kainulainen and V. Vihko

Division of Muscle Research, Department of Cell Biology, University of Jyväskylä, SF-40100 Jyväskylä 10 (Finland),
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Summary. Mice and rats were adjusted to daily treadmill training programs, which were heavy enough to increase the oxidative capacity of skeletal musles. Endurance training did not affect the activities of catalase and glutathione peroxidase and the concentration of vitamin E in the lungs of mice and rats. Thus increased ventilation and oxygen utilization induced by exercise training do not modify lung antioxidants, in contrast to hyperoxia and hypoxia.

Lung injuries appear in animals exposed to excessive concentrations of oxygen and other oxidants[2]. Hyperoxia increases H_2O_2 release by lung mitochondria and microsomes[3] and thus may cause peroxidative injuries. Lung tissue adapts to oxidant stress by increasing the level of antioxidants such as vitamin E, or the activities of catalase, glutathione peroxidase and superoxide dismutase[4,5]. On the other hand, exposure to hypoxia affects the antioxidant capacity of lung and causes structural changes and a tolerance to hyperoxia[6].

Some studies using expired pentane as the measure of lipid peroxidation have shown that physical exercise increases pentane expiration[7,8]. The real origin of this lipid peroxidation product has not been demonstrated. Physical exercise increases pulmonary ventilation and O_2 utilisation. Some studies also suggest that increased physical activity may induce structural changes in the lung tissue[9]. Hence, we studied whether endurance training modulates the level of some lung antioxidants.

Materials and methods. Male NMRI-mice, aged 4 months, were trained on a motor-driven treadmill (6° uphill tracks) once a day for 1 h at a speed of 25 m/min. A group of mice were killed the next day after 3, 10, and 20 training sessions. Male Sprague-Dawley rats, aged 8–9 weeks at the beginning of the study, were trained on a motor-driven treadmill with horizontal tracks. The daily running time and speed were gradually increased so that after 4 weeks' training the rats run for 1 h/day during 5 days a week. The running speed of each session was increased from 20 to 36 m/min for the last 30 min of the session. The total duration of the protocol was 6 weeks. The effect of the termination of regular endurance training (detraining) was also studied in this experiment. The first group of rats was killed the day after the last training session, the second 2 weeks after the cessation of training and the third group 5 weeks after the withdrawal of training.

The lungs were removed, washed in homogenization buffer (0.1 M potassium phosphate, pH 7.4), and trimmed free of connective tissues and major vessels. The activities of catalase and glutathione peroxidase and the concentration of vitamin E were assayed as described earlier[10] after modifying the reaction conditions optimal for lung tissue. Hydrogen peroxide was used as the substrate for both enzymes. The significance of the difference in the means between groups were tested by Student's t-test.

Results and discussion. Endurance training (commencement, prolonging, or termination) did not affect the activities of catalase and glutathione peroxidase and the concentration of vitamin E in the lungs of mice (table 1) and rats (table 2). The training programs were heavy enough to induce statistically

Table 1. Endurance training and the level of some antioxidants in mouse lung

Variable	Control	Endurance training (days)		
		3	10	20
	(n = 9)	(n = 9)	(n = 8)	(n = 8)
Catalase	13.9 ± 0.5	14.3 ± 0.6	15.0 ± 0.9	14.2 ± 0.6
Glutathione peroxidase	14.6 ± 0.5	15.1 ± 0.2	14.8 ± 0.7	14.7 ± 0.6
Vitamin E	19.9 ± 1.4	18.6 ± 1.1	21.5 ± 0.9	20.0 ± 2.0

Values are means ± SE. Catalase activity is expressed as arbitrary units x g^{-1} wet weight. Glutathione peroxidase activity is given as μmol x min^{-1} x g^{-1} wet weight. Vitamin E concentration is given as μg/g wet weight.