

Original article

Effect of rabbit line on a program of cryopreserved embryos by vitrification

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Abstract — The aim of this study was to assess the application of a cryopreservation program to preserve two selected rabbit lines. One of them was selected by litter size at weaning, line V (Synthetic breed). The second, line R (synthetic breed), was selected by growth rate. In this study, embryos were collected, from donor does belonging to the 7th and 15th generations of lines R and V, respectively, were vitrified and were stored from 1992–1993. Those embryos from donor does belonging to the 17th and 21st generations of lines R and V respectively, were vitrified and stored from 1998–1999. Embryo transfers were carried out in 1999. Morphologically normal embryos at the morulae stage were cryopreserved by vitrification in a 2.8 M dimethyl-sulfoxide + 3.5 M ethylene-glycol + 0.3 g·L⁻¹ bovine serum albumine in Dulbecco phosphate buffered saline solution. The main problem in the cryopreservation program was the low embryo production efficiency: significant differences were obtained in recovery efficiency between lines and line R showed the lowest proportion of donor does with 55% (at least 4 normal embryos) vs. 72% in line V. However, after transfer in recipient does of line V, the fertility rate at birth (81%), the rate of alive born by pregnant recipients (43%) and the number of males and does with offspring (9 to 18 different males, 12 to 32 females) enabled the different generations from each line to be re-established and studies on the selection process genetic gain to be developed.

cryopreservation / embryo / line / rabbit

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1. INTRODUCTION

Since the first cryopreservation study in the rabbit, the best embryo survival rates have been obtained at the morula stage [1–4]. Rabbit morulae seem to tolerate osmotic changes and the toxicity of cryoprotectants. This embryo stage allows the conventional freezing procedures to be substituted efficiently by a simple method of vitrification. Vitrification of rabbit morulae has been achieved by Smorag et al. [3] and Kobayashi et al. [5] with a mixture of propylene glycol and glycerol; however, the survival rates *in vivo* in these studies were relatively low (27 and 18%, respectively). Kasai et al. [6] and Vicente et al. [7] reported the best results in terms of live offspring after transferring cryopreserved embryos (65% and 56%, respectively); the former used a mixture of ethylene glycol and two non-permeating cryoprotectants (sucrose and ficoll) and the latter used a mixture of ethylene-glycol and dimethylsulfoxide.

Most studies carried out on cryopreservation of embryos gave *in vitro* or *in vivo* survival rates based on the transfer of a pool of cryopreserved embryos from various donor females. However, from a genetic point of view, in terms of re-establishing a line or strain, the number of donors with offspring and the offspring identification are important. Because of this, it is necessary to ensure embryo recovery from the greatest number of donor does and to define both optimal cryopreservation and transfer methods.

On the contrary, current research methods related to embryo cryopreservation have focused on technical factors which affect the process efficiency, but very few comparative studies have been done on the interactive relationship between technical factors and animal genotype (in rabbits [8, 9], in mice [10–12]).

The aim of this study was to assess the application of a cryopreservation program to preserve two selected rabbit lines.

2. MATERIALS AND METHODS

2.1. Experimental animals

Adult rabbits from two selected lines were used in this study. The Line V (synthetic breed) has been selected since 1980 on litter size at weaning. The second line, line R (synthetic breed), has been selected since 1990 on growth rate from weaning to slaughter age (28–63 day of age). Lines and selection methodologies applied were described by Estany et al. [13] and Estany et al. [14].

In this study, embryos were collected and vitrified in 1992–1993 and 1998–1999 from donor does belonging to the 7th and 17th generations of lines R and, 15th and 21st generations of lines V, respectively. The embryos used in this experiment were stored until their transfer in 1999.

2.2. Embryo recovery

Multiparous rabbit does (4th–6th parities) were mated to bucks from different reproductive groups (families) of the same line at the ratio of 3 does per buck. After 70–72 hours post-coitum, the donor does were slaughtered. The reproductive tract was then removed and the embryos were recovered by flushing with Dubelcco PBS (DPBS) at room temperature (20–25 °C). After recovery, morphologically normal embryos (morulae with both intact and regular mucin coat and zona pellucida, and homogenous cell mass) from each donor doe were washed twice in fresh DPBS supplemented with 0.002 g·mL⁻¹ BSA or 10% rabbit serum, and kept at room temperature until use (10 to 15 min). The following parameters were recorded in the donor does: ovulation rate estimated as the number of luteinized follicles with ovulation stigmas, the number of oocytes and the number of abnormal and normal embryos according to morphological criteria.

2.3. Vitrification procedure

A thousand one-hundred and twenty-seven morulae from lines V (15th and 21st generations) and R (7th and 17th generations) were vitrified.

The cryoprotective solution consisted of 1:1:2 (vol/vol/vol) of dimethyl-sulfoxide (3.5 M DMSO), ethylene glycol (4.4 M EG) and DPBS supplemented with 0.002 g·mL⁻¹ BSA or 10% rabbit serum [15].

Vitrification was carried out in two steps. First, normal embryos were pipetted into 0.2 mL of DPBS medium and placed in a glass culture dish and then 0.2 mL of the cryoprotective solution in DPBS was added and then diluted quickly, gyrating manually. The embryos were left in this medium for 2 min. In the second step, 0.6 mL of the cryoprotective solution was added and quickly diluted, yielding a final concentration of 2.8 M DMSO and 3.5 M EG in DPBS with a BSA supplement. Then the embryos were loaded into plastic mini-straws (IMV, L'Aigle, France) and were plunged directly into liquid nitrogen. Exposure time of the embryos to the final vitrification solution did not exceed 1 min. The two vitrification steps were carried out at 20 °C.

The straws contained three sections separated by air bubbles. The first consisted of PBS in the cotton plug, the second section the embryos suspended in the vitrification medium (about 0.1 mL) and placed in the middle of the fraction. The third section consisted of PBS. The straws were sealed with colored plastic. Each straw held between 6 and 14 normal compacted morulae or early blastocysts from each donor doe. Two straws per donor doe were used when the number of recovered embryos was higher than 14 normal embryos.

Devitrification was performed by immersing the second and third sections of the straws in a water bath at 20 °C for 10–15 s. The cryoprotective solution was removed from the embryos in a two-step dilution

procedure at room temperature (20–25 °C). The embryos suspended in the final vitrification solution (0.1 mL) were released into a glass dish containing 1 mL of 0.33 M sucrose in DPBS medium. After 2 minutes, the embryos were washed twice in fresh DPBS medium and scored morphologically before transfer. Only embryos with a homogenous cell mass and an intact mucin coat and zona pellucida were transferred.

2.4. Embryo transfer

One-hundred-thirteen recipient does were used. In accordance with previous studies [16], all recipient does were multiparous does belonging to line V and ovulation was induced in receptive does (red vulvar lips) 60–64 h before transfer with an intramuscular dose of 0.8 µg buseriline acetate (Hoescht).

Embryo transfer in does from a growth line such as R was inadvisable because their reproductive performance is low related to maternal line V (about a 52% birth rate, a litter size and mortality at birth about 7.6 and 15% respectively from line R and 78%, 10.2 and 8% respectively from line V [17, 18]).

The recipients were anesthetized by an injection of ketamine solution at the rate of 1.2 mL·kg⁻¹ body weight. Oviductal embryo transfer was performed unilaterally. Only donor does with more than six embryos stored were transferred in this study. Six to fourteen normal embryos were transferred to each recipient doe.

Not all donor does belonging to the last generation in each line were used to re-establish the population.

2.5. Assessment of viability

In vivo survival was assessed based on birth rates, the number of total offspring born and the number of live offspring.

2.6. Statistical analysis

The effect of strain and generation in each strain on embryo recovery data: the ovulation rate, normal embryos in donor does with at least four normal embryos and recovery rate (number of normal embryos recovered in donor does with at least four normal embryos/ovulation rate-) were analyzed by means of GLM [19]. The analysis of the recovery rate was performed using an arcsine angle data transformation.

A Chi-squared test with Yate correction was used to analyze the percentages of donor does (does with at least four normal embryos/total donor does), transferable embryos (non-damaged embryos after thawing/vitrified embryos), and birth rate, total (died + alive) and live-born rates on the transferred embryos, and cryopreservation efficiency (alive born/vitrified embryos) between the lines and generation in each line.

3. RESULTS

Table I shows the efficiency of the recovery rate in these lines conducted by mating. A total of 212 does were used as donors to provide embryos, 90% (190) of the

donors ovulated, the ovulation rate being 13.3 ± 0.18 without significant differences between the lines or the generations. However, the proportion of donor females with, at least, four normal embryos (65%) was greater in line V than in line R (72 vs. 55%, $P < 0.05$). The recovery failures can be classified as failures of the ovulation induction (10%), total recovery failure (9%) and fertilization failures (16%). The number of normal embryos and recovery rate per donor females were similar between the generations and the lines (9.9 ± 0.23 and $75 \pm 2\%$, respectively, Tab. I). The minimum and maximum number of normal embryos stored from a donor doe was four and twenty-three, respectively. Only 9 donor does had between 1 and 3 normal embryos (5 and 4 for lines V and R) and they were not vitrified and stored.

For line V, at least one straw was preserved from 23 males of different 15th generation reproductive groups and 24 males from the 21st generation. Meanwhile, for line R at least one straw was stored from 9 and 10 different males from the 7th and 17th generations, respectively.

A primary problem in the cryopreservation procedures was the proportion of intact embryos (homogeneous cell mass and zona

Table I. Embryo recovery from two selected rabbit lines.

	Total does	Ovulating does	Ovulation rate LSM \pm SE	Donor does (%)	¹ Recovery rate LSM \pm SE	² Normal embryos LSM \pm SE
V15	67	58 (87)	13.3 0.35	48 (73)	73 3	9.8 0.45
V21	56	48 (86)	13.1 0.39	40 (71)	76 3	9.8 0.50
Vtotal	123	106 (86)	13.2 0.26	88 (72) ^a	74 2	9.8 0.36
R7	32	28 (88)	14.4 0.51	18 (59)	81 5	11.2 0.74
R17	57	56 (98)	13.0 0.36	31 (54)	73 4	9.7 0.56
Rtotal	89	84 (94)	13.5 0.30	49 (55) ^b	77 3	10.2 0.48
Total	212	190 (90)	13.3 0.18	137 (65)	75 2	9.9 0.23

LSM \pm SE: least square means \pm standard error.

^{a,b} Values in the same column with different superscripts are statistically different $P < 0.05$.

¹ Data showed in Table I without angular transformation.

² Normal embryos: normal embryos in donor does (does with at least 4 normal embryos).

Table II. Cryopreservation efficiency from two selected rabbit lines.

	Vitrified embryos (No. donor does)	Transferable embryos (%)	Recipient does	Fertility at birth (%)	Total born (%)	Alive born (%)	Cryopreservation efficiency (%)
V15	407 (43)	345 (85)	41	32 (78)	151 (53)	136 (48)	33
V21	338 (34)	303 (90)	34	32 (94)	129 (45)	120 (42)	36
VTotals	745 (77)	648 (87) ^a	75	64 (85)	280 (49)	256 (45)	34 ^a
R7	194 (18)	155 (80)	16	13 (81)	55 (40)	50 (36)	26
R17	188 (22)	159 (85)	22	14 (64)	46 (43)	45 (43)	24
Rtotal	382 (40)	314 (82) ^b	38	27 (71)	101 (42)	95 (39)	25 ^b
Total	1127 (117)	962 (85)	113	91 (81)	381 (47)	351 (43)	31

^{ab} Values in the same column with different superscripts are statistically different $P < 0.01$. Total and alive born (%): the percentage was calculated on transferred embryos in pregnant does. Cryopreservation efficiency (%): percentage of alive born on vitrified embryos.

pellucida and mucin coat without damage) after devitrification or thawing; line R showed a smaller percentage of intact embryos, 82% of 382 vitrified embryos were scored as intact embryos vs. 87% of 745 vitrified embryos of line V ($P > 0.05$, Tab. II).

All transfers were carried out in females belonging to line V, obtaining a fertility rate at birth of 85% and 71% for vitrified embryos from line V and line R, respectively. Both total and alive-born rates were slightly higher for the vitrified embryos belonging to the maternal line V than for growth line R, but these differences were not statistically significant (49% and 45% vs. 42% and 39%, respectively, Tab. II). The mean number of alive-born by pregnant recipients was 3.9 ± 0.22 (4.1 ± 0.25 and 3.7 ± 0.39 for lines V and R, respectively, data not shown in the tables). Cryopreservation efficiency, live born per vitrified embryos was higher in line V than in line R (34% vs. 25%, $P < 0.01$, Tab. II).

No significant differences were observed between the generations of the lines V and R for the variables analyzed.

In relation to the number of males of the different reproductive groups with at least one offspring, 14 and 18 different males had offspring from the 15th and 21st generations of line V, respectively. Meanwhile, 9 different males had offspring from both generations of line R.

4. DISCUSSION

In this work, lines V and R were used to assess the global efficiency of a cryopreservation program by vitrification (Tabs. I and II). Finally, the aim was to re-establish the population from two generations of each line in order to evaluate the genetic gain for the selection characters, litter size at weaning to lines V and A, and growth rate during the fattening period to line R. The efficiency depends on technical factors (ovulation induction, flushing and scoring embryos, cryopreservation, storage and transfer procedures) as well as genetic factors (donor and recipient effects).

The differences between lines in transferable embryos and cryopreservation efficiency (live-born at birth/vitrified or frozen

embryos) were in accordance with the results from previous studies carried out to define the optimal freezing procedure [7, 20]. We had observed differences in the tolerance to cryoprotectants and freezing procedures in accordance with the studies in a mouse line of Schmidt et al. [12]. Recently, García et al. [20] observed differences in the survival rate at birth between vitrified embryos belonging to lines V and R (43% vs. 22%, respectively). The survival rates obtained in this study were better than those obtained by García et al. [20], probably due to the recipient genotype; in this work, we transferred the vitrified embryos in a pool of recipient does of undefined genetic origin in a Uruguayan rabbit farm. This last aspect is another important factor in rabbits assessed by Vicente and García-Ximénez [9] and Joly et al. [21]. To obtain high fertility and survival rates, line V and line 1077 seemed to be better recipients than the lines which have New Zealand White origin.

This study showed that it is possible to use vitrification as a method to cryopreserve rabbit embryos from two selected lines rapidly and efficiently after six years of storage. In the studies by Joly [22], it was demonstrated that an effective preservation of characteristics such as growth rate and litter size could be obtained with the offspring of 9 males from different reproductive groups (9 fathers) for a heritability of these characters of 0.25 and 0.10 and variation coefficients of 0.10 and 0.35, respectively. In accordance with the number of different males and does with offspring after cryopreservation, the values of the effective population size (N_e) for maternal line (V) were higher than 70 and for growth line (R) about 46, so it was possible to re-establish the lines with an increment of consanguinity coefficient around 1% and it was possible to assess the genetic gain of the selection process.

However, embryo production efficiency was the limiting factor to establishing an embryo bank. An important group of donor does (35%) did not give normal embryos

due to both non-ovulation induction (10%), oocyte fertilization failures (16%) and total recovery failures (9%). This problem was especially important in line R (45%). The buck effect is important in ovulation induction and fertilization; although the bucks had been previously tested, the use of artificial insemination could reduce the percentage of failure by non-ovulation induction and fertilization. In fact, this technique is now being used to conduct the population of line R and to recover embryos to constitute an embryo bank.

In this study, no hormonal treatment was used to increase the average number of normal embryos because, if the cryopreservation procedure is optimum, the number of recovered embryos per donor in commercial selected lines is enough to obtain about 4 pups per donor doe (4.1 for line V, 3.7 for line R). Previous works on superovulation treatment showed a reduction in families or reproductive groups represented in the embryo bank (-17% donor does [23]), although the mean number of embryos stored by the donor increased [21]. In addition, several authors have observed a negative effect of the superovulation treatment on the survival rate of rabbit embryos [24]. An improvement in superovulation treatments and the use of the laparoscopic technique in multiple embryo collection *in vivo* [25] may be useful to facilitate the reconstitution of lines or races similar to the R line.

Some of the applications of the cryopreservation program tested in this study have been to establish a hyperprolific line in the rabbit thus increasing the selection pressure on the live-born character [26], to export embryos from selected animals [20] and more recently to evaluate the genetic gain of line V [27].

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REFERENCES

- [1] Bank H, Maurer RR. Survival of frozen rabbit embryos. *Exp Cell Res* 1974, 89: 188–196.
- [2] Tsunoda Y, Sugie T. Survival of rabbit eggs preserved in plastic straws. *J Reprod Fertil* 1977, 49: 173–174.
- [3] Smorag Z, Gajda B, Wieczorek B, Jura J. Stage-dependent viability of vitrified rabbit embryos. *Theriogenology* 1989, 31: 1227–1231.
- [4] Lopez-Bejar M, López-Gatius F, Camon J, Rutllant J, Labernia J. Development in vitro of rabbit embryos after freezing by two-steps or ultrarapid cooling methods. *J Vet Med A* 1994, 41: 780–790.
- [5] Kobayashi K, Nagashima H, Yamakawa H, Kato Y, Ogawa S. The survival of whole and bisected rabbit morulae after cryopreservation by the vitrification method. *Theriogenology* 1990, 33: 777–788.
- [6] Kasai H, Hamaguchi Y, Zhu SE, Miyake T, Sakurai T, Machida T. High survival of rabbit morulae after vitrification in an Ethylene Glycol-Based solution by a simple method. *J Reprod Fertil* 1992, 89: 91–97.
- [7] Vicente JS, García-Ximénez F. Osmotic and cryoprotective effects of mixture of DMSO and ethylene glycol on rabbit morulae. *Theriogenology* 1994, 42: 1205–1215.
- [8] Maurer RR, Haseman JK. Freezing morula stage rabbit embryos. *Biol Reprod* 1976, 14: 256–263.
- [9] Vicente JS, Garcia-Ximénez F. Effects of two cryoprotective agents used in slow or rapid standard freeze-thaw procedure on the in vivo survival of morulae from three rabbit strains. *INIA* 1993, 8: 247–256.
- [10] Pomp D, Eisen EJ. Genetic control of survival of frozen mouse embryos. *Biol Reprod* 1990, 42: 775–786.
- [11] Schmidt MP, Hansen CT, Wildt DE. Viability of frozen-thawed mouse embryos is affected by genotype. *Biol Reprod* 1985, 32: 507–514.
- [12] Schmidt MP, Schiewe MC, Wildt DE. The genotypic response of mouse embryos to multiple freezing variables. *Biol Reprod* 1987, 37: 1121–1128.
- [13] Estany J, Baselga M, Blasco A, Camacho J. Mixed model methodology for estimation of genetic response to selection in litter size in rabbits. *Livest Prod Sci* 1988, 21: 67–75.
- [14] Estany J, Camacho J, Baselga M, Blasco A. Selection response of growth rate in rabbits for meat production. *Genet Sel Evol* 1992, 24: 527–537.
- [15] Vicente JS, Viudes-De-Castro MP, García ML. In vivo survival rate of rabbit morulae after vitrification in a medium without serum protein. *Reprod Nutr Dev* 1999, 39: 657–662.
- [16] Vicente JS, García-Ximénez F. Effect of recipient doe genotype on survival rate at birth of frozen embryos. *Reprod Nutr Dev* 1993, 33: 229–234.
- [17] García ML, Baselga M, Vicente JS, Lavara R. Selection response on reproductive characters in a maternal line of rabbits. *Proc. 7th World Rabbit Congress. 2000, Vol. A, p 381–387.*
- [18] Lavara R, Viudes-de-Castro MP, Vicente JS, Mocé E. Effect of synthetic prostaglandin F_{2a} analogue (cloprostenol) on litter size and weight in a rabbit line selected by growth rate. *World Rabbit Sci.* 2002, 10: 1–5.
- [19] SAS, Statistical Analysis System, copyright 1989–1996. SAS Institute, Inc.
- [20] García ML, Blumetto O, Capra G, Vicente JS, Baselga M. Vitrified embryos transfer of two selected Spanish rabbit lines to Uruguay. *Proc. 7th World Rabbit Congress. 2000, Vol. A, p 139–142.*
- [21] Joly T, Vicente JS, Theau-Clément M, García-Ximénez F, Besenfelder U, Renard JP. Cryopreservation of genetic resources in rabbit species: practical application. *Proc. 6th World Rabbit Congress. 1996, Vol. 2, p 293–298.*
- [22] Joly T. Etablissement d'une cryobanque de semence ou d'embryons pour la conservation ex situ de la diversité génétique chez les mammifères domestiques: L'exemple du lapin (*Oryctolagus cuniculus*). Thesis, Institut National des Sciences Appliquées de Lyon, 1997.
- [23] García-Ximénez F, Vicente JS. Effect of PMSG treatment to mating interval on the superovulatory response of primiparous rabbits. *J Appl Rabbit Res* 1990, 13: 71–73.
- [24] Renard JP, Garnier V, Parvex R. Production et congélation de l'embryon de lapin. *Proc. 3^{es} Journées de la Recherche Cunicole. 1982. Communication n° 18.*
- [25] Besenfelder U, Strouhal C, Brem G. A method for endoscopic embryo collection and transfer in the rabbit. *J Vet Med A* 1997, 45: 577–579.
- [26] Cifre J, Baselga M, Garcia-Ximenez F, Vicente JS. Performance of a rabbit line founded selecting on hyperprolificacy I. Litter size traits. *J. Anim. Breed. Genet.* 1998, 115: 131–138.
- [27] García ML. Evaluación de la respuesta a la selección en dos líneas maternas de conejo, Thesis, Polytechnic University of Valencia, 2001.

