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Original article

Effects of strain and embryo transfer model (embryos from one *versus* two donor does/recipient) on results of cryopreservation in rabbit

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Summary — Differential effects of 2 transfer models for normal thawed embryos of 1 donor doe were studied on the offspring rate and their embryo survival at birth from 3 selected rabbit strains (SY and SB: synthetic strains, NZ: New Zealand White). Morulae were obtained 64-66 h post-coitum from 93 adult does treated with 25 IU of hCG (SY:36, NZ:27, SB:30). Morphologically normal morulae were frozen in the presence of 1.5M DMSO and stored in liquid nitrogen. Normal thawed embryos were transferred into the oviducts of synchronized recipient does of the same strain 48 h after being injected with 25 IU of hCG (SY:28, NZ:21, SB:24). Each recipient received embryos from 1 (single transfer) or 2 different donor does (double transfer). Significant differences were observed in the post-thawing percentage of normal embryos between strains (SY: 95 ± 1% and SB: 85 ± 3%, $P < 0.05$; NZ: ,91 ± 2%). After transfer, no significant differences were observed in pregnancy rate and offspring rate between the transfer models, whereas significant differences were only found in survival rate when all transfers were analyzed (double: 24 ± 4% *vs* single: 14 ± 3%, $P < 0.05$). An effect of strain was detected in the pregnancy rate (NZ: 33% *vs* SB: 71%, $P < 0.05$; SY: 61%) and in the survival rate per donor doe on pregnant recipient doe (SY: 42 ± 5 *vs* SB: 19 ± 5, $P < 0.05$; NZ: 34 ± 7%). These results suggest a differential embryo sensitivity with respect to their genetic origin in both the freezing-thawing and transfer procedures.

cryopreservation / embryo / transfer / rabbit strain

Résumé — Effets des souches et des modèles de transfert d'embryons sur les résultats de cryopréservation des embryons de lapin. *L'étude des effets de deux modèles de transfert d'embryons de lapin a été réalisée sur trois souches sélectionnées (SY et SB : souches synthétiques, NZ : néozélandaise). Les résultats sont exprimés par les pourcentages de femelles donneuses obtenant des descendants et le nombre de leurs embryons survivants à la naissance. Les embryons au stade morula sont obtenus 64 à 66 heures post coitum à partir de 93 femelles adultes traitées avec 25 UI d'hCG (SY : 36, NZ : 27, SB : 30). Les embryons morphologiquement normaux sont congelés en présence de 1.5 M de DMSO et stockés dans de l'azote liquide. Les embryons intacts au dégel sont transférés dans l'oviducte de femelles receveuses de la même souche synchronisées 48 h plus tôt, par injection de 25 UI d'hCG (SY : 28, NZ : 21, SB : 24). Chaque receveuse reçoit les embryons soit d'une seule lapine donneuse (transfert simple), soit de deux donneuses différentes (transfert double). Au dégel, des différences significatives sont observées dans les pourcentages d'embryons normaux selon les souches (SY : 95 ± 1% and SB : 85 ± 3%, P < 0.05; NZ : 91 ± 2%). Après transfert, aucune différence significative du taux de gestation et du taux de descendants n'est observée*

entre les modèles de transfert. Par contre, le taux d'embryons survivants présentent une différence significative selon les modèles : double, 24 ± 4%, vs simple, 14 ± 3%, P < 0.05. Un effet de souche est observé sur le taux global de gestation (NZ : 33% vs SB : 71%, P < 0.05; SY : 61%) et le taux d'embryons suvivants chez les receveuses gravides selon les femelles donneuses (SY : 42 ± 5 vs SB : 19 ± 5, P < 0.05; NZ : 34 ± 7%). Ces résultats suggèrent une sensibilité différente des embryons, en fonction de leur origine génétique, aux processus de congélation-décongélation et de transfert.

cryopréservation / embryons / transfert / lapin / souches

INTRODUCTION

Embryo banks are valuable tools in the livestock improvement schemes where a control population is needed to measure the actual rate of genetic gain (Polge, 1977). In such genetic experiments it is important to ensure an even representation of the parental population to obtain offspring from the greatest number of donor females.

The most important phases of a cryopreservation program are: embryo recovery, freezing-thawing and transfer of embryos. In a previous work, we studied the recovery (Vicente and García-Ximénez, 1991) and freezing of embryos in rabbits (García-Ximénez and Vicente, 1991). In the present paper, our aim was to study the differential effects of 2 models for embryo rabbit transfer (single: thawed embryos from 1 donor/recipient; and double: thawed embryos from 2 donors/recipient) on the percentage of donor does which produce offspring and their embryo survival rate at birth in 3 selected rabbit strains.

MATERIALS AND METHODS

Rabbit does of 3 strains were used in the experiment. Two strains had been selected on litter size at weaning, strain NZ (White New Zealand, 13th generation) and strain SY (Synthetic breed, 10th generation). The third strain SB (2nd generation) had been selected on growth rate from weaning to slaughter (28–77-d of age). This strain is a synthetic breed derived from 2 strains previously selected for high growth rate. These strains and selection methodologies applied were described by Estany *et al* (1988).

One hundred and sixty-six does, kept individually under the same environmental conditions (16 h light: 8 h dark, 20–25 °C) were used; 93 as embryo donors, 73 as recipient does.

Early morulae were obtained from adult does (SY : 36, NZ : 27, SB : 30) naturally mated with males of the same strain. Immediately after mating, all donor does were injected intravenously with a dose of 25 IU hCG (Coriogan, Ovejero). Does were killed 64–66 h after mating. The reproductive tract was immediately removed and morulae were recovered by flushing each oviduct at room temperature with 3 ml of Dulbecco's phosphate-buffered saline (PBS, Sigma) containing 50% heat-inactivated rabbit serum (Vicente and García-Ximénez, 1991). The embryos were washed once in the same medium at room temperature and scored according to morphological criteria (Carney and Foote, 1990). Only normal morulae were frozen. Ovulation rate (estimated from the number of partially formed corpora lutea), number of recovered embryos and normal embryos were noted. The freezing and thawing procedures used were derived from those described by Tsunoda *et al* (1982) and Kojima *et al* (1985 and 1987). The normal embryos recovered from each donor doe (10.7 ± 0.2) were pipetted into 0.2 ml of PBS containing 50% heat-inactivated rabbit serum in an embryological watch glass. Then, 0.3 ml of 2.5 M of dimethyl sulphoxide (DMSO, Panreac) in PBS was added in 3 steps of 0.1 ml at 5-min intervals at room temperature. The final concentration of cryoprotectant was 1.5 M. All normal morulae from 1 donor doe suspended in 1.5 M of cryoprotectant were loaded into one 0.25 ml-capacity plastic artificial insemination straws. Each straw was identified and sealed with a col-

oured plastic rod. The straws were then placed in the cooling chamber of a programmable biological freezer (Kryo-10 1.7, Planer). Cooling rate from initial temperature (+ 20 °C) to –5.3 °C was –1 °C/min. The straws were then cooled at –0.1 °C/min until –7 °C (equilibrium ramp). The automatic seeding was programmed at –5.5 °C for 3 min. After equilibrium ramp, the straws were frozen at –1 °C/min to –80 °C and between –80 °C and –120 °C at –8 °C/min before plunging in liquid nitrogen. All embryos were stored at –196 °C for several days. The thawing of embryos was achieved in air at room temperature (25 °C) for 3 min. The content of the straws was expelled into a embryological watch glass containing 2 ml of PBS. The thawed embryos were washed in fresh PBS at room temperature and counted. Their morphology was examined before transfer; only morulae with normal blastomeres and with undamaged zona pellucida and mucin coat were classified as transferable.

The normal frozen-thawed embryos were transferred into the oviducts of 73 pseudopregnant nulliparous does which received an intravenous injection of 25 IU hCG 48 h before the transfer (SY: 28, NZ: 21, SB: 24). This asynchronous transfer of frozen-thawed rabbit morulae was carried out according to Tsunoda *et al* (1982); García-Ximénez and Vicente (1991). None of the transferred recipients had ovarian cystic or haemorrhagic follicles (known to have a negative effect on fertility: García-Ximénez and Vicente, 1992). The recipient does were anaesthetized by im injection of 5:1 ketamine chlorohydrate (Ketolar 50 mg/ml, Parke-Davis): prometazine (Phenergan 10 mg/ml, Rhone-Poulenc) solution (1.2 ml/kg body weight) followed 5 min later by iv injection of 1.5 ml of the same solution in the marginal ear vein. Transfers were carried out by a midline ventral laparatomy. The embryos syspended in PBS were held in a glass tube (1 mm external diameter and 0.5 mm internal diameter) connected to a 1-ml insulin syringe. The glass tube was inserted through the infundibulum (0.5 cm) into the oviduct and the embryos carefully placed.

Each recipient hosted embryo from 1 or 2 different donor does of the same strain. The transfer models were:

– "Single": all normal embryos thawed from only one donor doe were transferred into the oviducts of a recipient doe (half on each side);

– "Double": all normal embryos thawed from 2 donor does were separately transferred into the oviducts of a recipient doe (one set on each side).

This implies that the mean number of embryos transferred to each recipient was twice as high in "double" (8.4 ± 0.5 per uterine horn) than in "single" transfer (9.4 ± 0.4 per recipient doe).

The pregnancy of recipient does was diagnosed by abdominal palpation 12 d after ovulation induction (Adams, 1982). The recipient does which became pregnant in the "double" transfer group were slaughtered on the 29th d after hCG injection, each uterine horn was opened to evaluate the number of live foetuses. In the pregnant recipients of the "single" transfer group, the number of live foetuses was evaluated at birth.

Analysis of data

Embryo recovery and freezing

The ovulation rate, the number of total recovered embryos, the percentage of normal recovered embryos (normal recovered embryos/total recovered embryos per donor doe) and the percentage of normal thawed embryos (normal thawed embryos/frozen embryos per donor doe) among strains were analyzed by an analysis of variance. Protected LSD test was used to compare means (Snedecor and Cochran, 1980).

Viability assessment

A χ^2 test was used to analyze the pregnancy rate (recipient does with gestation to term/total recipient does) and offspring rate (donor does with offspring/donor does from which there were transferred embryos) between transfer models and strains.

The following parameters of survival rate were established:

– survival rate per donor doe evaluated on all recipient does (SR1); – survival rate per donor doe evaluated on pregnant recipient does (SR2); – survival rate evaluated on donor doe with at least 1 live fetus or pup at birth (SR3).

Variation between donor does was not ignored (Pomp and Eisen, 1990), and the analysis of survival rate as performed on data from individual donor does, leading to a more conservative test of genotypic differences.

Analysis of variance after arcsine transformation of the survival rate at birth (live fetuses at birth or at slaughter/transferred embryos) was used to evaluate the effects of strain and transfer model and their interactions. Protected LSD test was used to compare means of SR1, whereas the Tukey test was used to compare means of SR2 and SR3.

RESULTS

Recovery and freezing-thawing of embryos

No significant differences were found in the ovulation rate (m ± SEM: 12.9 ± 0.4, 13.1 ± 0.5 and 14.1 ± 0.5), in the number of recovered embryos (10.6 ± 0.5, 10.7 ± 0.6 and 11.6 ± 0.6) and in the percentage of morphologically normal embryos (98 ± 1%, 98 ± 1% and 97 ± 1%) among the strains (SY, NZ and SB, respectively). The overall mean number of recovered embryos was 11 (82% as a percentage of the ovulation rate). After freezing–thawing procedures, significant differences were observed in the percentage of normal thawed embryos among the strains ($P < 0.05$). The percentage of normal thawed embryos from the SB strain was lower than from the SY strain (85 ± 3% *vs* 95 ± 1%), whereas that for NZ strain was 91 ± 2%.

Pregnancy and offspring rates (table I)

The observed differences in the overall pregnancy and offspring rates between transfer models were not statistically significant (51 and 38% in single transfer *versus* 70 and 53% in double transfer, respectively). However, when the pregnancy rate was evaluated among the strains, significant differences were found. The NZ strain shows the lowest pregnancy rate (33%). In spite of the high pregnancy rate from the SB strain (71%), there were 7 pregnancy failures to term out of 12 recipient does diagnosed pregnant in the single model. In a previous work (not published), we observed a high post-placentation mortality in the SB strain (30%) when frozen embryos were transferred. In addition, none of the recipient does from single transfer gave birth to 1 pup, while only 1 live foetus was observed in 3 uterine horns of recipient does with frozen embryos from 2 donor does.

The average offspring rate was 44% (41/93) with no significant differences among the strains (SY: 58%, NZ: 33%, SB: 41%, $P > 0.05$).

Survival rate (table II)

Significant differences were only found in survival rate between transfer models and among strains when this was studied on all transfers performed (embryos from 93 donor does transferred to 73 recipient does). The survival rate (SR1) in the double transfer model was higher than in the single transfer model (24 ± 4% *vs* 14 ± 3%, $P < 0.05$) and in the SY strain was higher than the other 2 strains (SY: 27 ± 5% *versus* NZ: 13 ± 4% and SB: 14 ± 4%, $P < 0.05$). When the analysis of assessment of viability of donor does was restricted to pregnant recipient does (embryos from 55 donor does transferred on 41 recipient does), significant differences were found in the survival rate (SR2) among the strains (SY: 42 ± 5% *vs* SB: 19 ± 5%, $P < 0.05$, NZ: 34 ± 7%). The survival rate between transfer models did not differ statistically (27 ± 4% and 34 ± 5%, single and double transfer respectively). If the survival rate was analyzed restricted to the donor does with offspring (SR3), no significant differences were found between the transfer models and among the strains (single: 37 ± 4% and double: 46 ± 4%; SY: 46 ± 4%, NZ: 43 ± 6% and SB: 34 ± 5%). No significant in-

Table I. Effects of strain and transfer model on pregnancy rate and offspring rate.

	Single transfer				*Double transfer*				*Strain*			
	Recipient does	*Donor does*	*Pregnancy rate (%)*	*Offspring rate (%)*	*Recipient does*	*Donor does*	*Pregnancy rate (%)*	*Offspring rate (%)*	*Recipient does*	*Donor does*	*Pregnancy rate (%)*	*Offspring rate (%)*
SY	20	20	11 (55)	11 (55)	8	16	6 (75)	10 (63)	28	36	17 (61)^{ab}	21 (58)
NZ	15	15	4 (27)	4 (27)	6	12	3 (50)	4 (33)	21	27	7 (33)^{b}	9 (33)
SB	18	18	12 (67)	5 (28)	6	12	5 (83)	7 (58)	24	30	17 (71)^{a}	11 (41)
	53	53	27 (51)	20 (38)	20	40	14 (70)	21 (53)	73	93	41 (56)	41 (44)

Pregnancy rate: number of recipient does with gestation to term/total recipient does; offspring rate: number of donor does with offspring/number of donors does from which there were embryos transferred. ^{a,b} Values with different superscripts are statistically different ($P < 0.05$).

Table II. Effects of strain and transfer model on survival rate[1] per donor doe in all transferred recipients (SR1) and in pregnant recipient does (SR2) and survival rate from donor does with offspring (SR3).

	Single transfer				*Double transfer*				*Strain*			
	Transferred embryos [2]	*SR1*	*SR2*	*SR3*	*Transferred embryos* [2] *(per uterine horn of recipient)*	*SR1*	*SR2*	*SR3*	*Transferred embryos* [2]	*SR1*	*SR2*	*SR3*
SY	9.3 ± 0.6	20 ± 5	37 ± 5	37 ± 5	8.1 ± 0.6	34 ± 8	46 ± 8	55 ± 6	8.7 ± 0.5	27 ± 5[a]	42 ± 5[a]	46 ± 4
NZ	9.5 ± 0.6	10 ± 4	36 ± 5	36 ± 5	8.3 ± 0.6	16 ± 8	33 ± 12	49 ± 10	8.9 ± 0.5	13 ± 4[b]	34 ± 7[ab]	43 ± 6
SB	9.3 ± 0.5	10 ± 5	16 ± 7	38 ± 9	8.6 ± 0.8	18 ± 6	22 ± 8	31 ± 6	9.0 ± 0.4	14 ± 4[b]	19 ± 5[b]	34 ± 5
	9.4 ± 0.4	14 ± 3[b]	27 ± 4	37 ± 4	8.4 ± 0.5	24 ± 4[a]	34 ± 5	46 ± 4	8.9 ± 0.3	18 ± 2	31 ± 3	42 ± 3

Mean ± standard error of mean. 1 Survival rate (%): number of live fetuses or pups/number of transferred embryos per donor doe; 2 transferred embryos: mean of embryos transferred per donor doe. [a,b] Values with different superscripts in the same variable are statistically different ($P < 0.05$).

teractions were observed in survival rate (SR1, SR2 and SR3) between the factors (model and strain).

The SY strain showed better results in both transfer models, reaching the offspring rate 63% in double transfer (table I) and a survival rate of 55 ± 6% (live fetuses: SR3, table II).

DISCUSSION

The majority of studies carried out on cryopreservation of embryos, give *in vivo* rates after thawing based on the transference of a pool of frozen embryos which come from various does (Tsunoda *et al,* 1982; Kojima *et al,* 1985; Techakumphu and Heyman, 1987). However, from a genetic point of view, in terms of establishing a control group, the number of donor does with offspring and the offspring identification are important.

The first major step of a cryopreservation program is to recover normal embryos from all donor does. The percentage of normal recovered embryos was about 82% and the percentage of them morphologically normal was very high in the 3 strains (97 to 98%). The superovulation technique with eCG was not used because, in these conditions, normal embryos from 35% of donor does cannot be recovered (García-Ximénez and Vicente, 1990). The negative effect of superovulation (FSH o eCG) on embryo quality and recovery rate was already observed in rabbits (Fujimoto *et al,* 1974; Renard *et al,* 1982; Fisher and Meuser-Oderkichen, 1988; Schmidt *et al,* 1992).

During freezing-thawing procedures, normal embryos can be damaged. The application of morphological criteria to screen rabbit embryos after thawing is important, given that rabbit embryos without zona pellucida and mucin coat fail to develop after transfer (Moore *et al,* 1968; Rottmann and Lampeter, 1981). Our percentages of normal embryos after thawing in SY and NZ strains agree with those obtained in rabbits by previous authors (Tsunoda *et al,* 1982; Kojima *et al,* 1985, 1987; García-Ximénez and Vicente, 1991), but were lower in SB strains. In addition to the differences after thawing among strains, the survival rate at birth per donor doe in pregnant recipient does (SR2) was higher in the SY and NZ strains than in the SB strain. This suggests a differential sensitivity of embryos with respect to their genetic origin in both the freezing-thawing and transfer procedures. It is possible that our standard procedure for freezing-thawing and synchrony between embryos and recipients could be suboptimal for the SB embryos. Maurer and Haseman (1976) also found strain differences in freezing–thawing sensitivity of rabbit embryos and in maternal genotype of recipient doe. In mouse embryos, some authors have reported that post-thawing viability was influenced by maternal and embryonic genotype (Schmidt *et al,* 1985, 1987; Pomp and Eisen, 1990). Reciprocal transfers are thus necessary to evaluate the maternal effect on the low offspring rate of NZ and SB strains.

Comparing the 2 models of embryo transfer, it was observed that the single transfer has the risk that only 1 foetus might develop. In rabbit does, at least 2 foeto-placental units are required to maintain a gestation (Adams, 1962 and 1970). When survival rates are high in double transfers, competition between foetuses can lead to a higher foetal mortality (Adams, 1962; Johnson, 1971). Thus, total or partial losses of offspring from some donor does could be the consequence of defect or excess in the initial number of placentated embryos. The offspring rate was slightly higher in double transfer than single transfer (53 *vs* 38%). This difference, not statistically significant, could be due to some

pregnancy failures in the single transfers, because the initial number of implanted embryos was reduced.

The overall offspring rate obtained in the present work was 41% and the survival rate in these donor does with offspring 42%. These results can be favourably compared to those reported by Hatton *et al* (1985) with an embryo survival rate of 31% in pregnant recipient does (5 from 9 transferred does). In our case, only SY strain showed satisfactory results (offspring rate: 58% and survival rate: 46%). Moreover, when the frozen embryos were transferred by the double model (offspring rate: 63% and survival rate: 55%), results were similar to those obtained with transferred fresh morulae of this strain (Vicente and García-Ximénez, 1991) which also agree with results obtained by Hatton *et al* (1985) in the transfer of fresh morulae. Further studies are still necessary in the other 2 strains, SB and NZ, to evaluate the effects of embryonic and maternal genotypes on cryopreservation program.

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