EMBRYO TRANSFER AS A METHOD FOR ELIMINATING PATHOGENIC AGENTS IN A RABBIT COLONY

Toepassing van embryotransfer voor de eliminatie van pathogenen in konijnenstapels

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ABSTRACT

To regain the SPF status of a contaminated but genetically valuable rabbit breeding unit, embryos from the contaminated does were transferred into SPF recipient females. Embryos were collected on day 3 of gestation by flushing uterine horns. All usable embryos were frozen, and a portion of them were preserved in liquid nitrogen to form a stock of highly valuable genotypes. Thirty-two stimulated does produced 893 embryos, 821 (92%) of which had an intact zona pellucida and were cryopreserved. Of this stock, 478 embryos were thawed, 466 were recovered (97.5%) and 417 were of good quality (87.2%). In 30 does, 10 to 18 embryos were surgically transferred per recipient doe and 24.9% (104/417) of them developed to term after transfer. This corresponds to an average number of 3.47 (104/30) live newborns per recipient.

Health screenings performed on sanitized rabbits confirmed the disappearance of pathogenic agents.

SAMENVATTING

Om de SPF-status van een besmette maar genetisch waardevolle konijnenstapel te herwinnen, werden embryo's overgeplaatst van besmette voedsters naar SPF-draagvoedsters. De embryo's werden verzameld door spoeling van de baarmoederhoornen op dag 3 van de dracht. Alle bruikbare embryo's werden diepgevroren, een deel van hen werd bewaard in vloeibare stikstof en vormt zo een waardevolle genetische stock.

Embryo's, 893 in totaal, werden gewonnen uit 32 hormonaal gestimuleerde voedsters. 821 (92%) hadden een intacte zona pellucida en werden bewaard onder vloeibare stikstof. Uit deze stock werden 478 embryo's ontdooid, waarvan 466 (97,5%) konden gerecupereerd worden en 417 (87,2%) kwalitatief goed waren. Tien tot 18 embryo's per voedster werden chirurgisch ingeplant. Na de transfer werd 24,9% (104/417) van de embryo's levend geboren. Dit stemt overeen met een gemiddelde van 3,47 (104/30) levend geboren jongen per receptorvoedster.

Serologische screenings later uitgevoerd op deze konijnen, bevestigden de afwezigheid van alle pathogene agentia.

INTRODUCTION

Healthy animals are essential both for proper human food production and for successful research projects. Infections reduce farm production rates and may produce effects that influence the outcome of experiments. This is why animals are commonly bred and housed under conditions in which precautions are taken to avoid infections. The term "specific pathogen free" (SPF) is used to describe research animals. These have to be bred and maintained following the health recommendations of the FELASA (Working Group on Animal Health, 1994).

Valuable breeding rabbits were contaminated by different pathogenic agents such as *Encephalitozoon cuniculi*, *Clostridium piliformis* and Rotavirus.

Encephalitozoon cuniculi, an amitochondriate intracellular parasite of mammals which has an organotropism for brain, spinal cord, kidneys and liver. Tyzzer's disease is caused by *Bacillus piliformis,* which has recently been reclassified as *Clostridium piliformis*. This organism is a gram-negative, intracellular, pleomorphic, spore-forming bacterium. Animals die due to lesions on the liver and the lower intestine. Rotavirus infects mostly young rabbits and may be responsible for diarrhea. The virus is transmitted horizontally and is endemic in most rabbitries.

Two methods are available for restoring the health status of a rabbit colony: derivation by aseptic hysterectomy and embryo transfer. Aseptic hysterectomy consists of a cesarean section of a full-term pregnant female. Following transfer of the uterus through a germicide bath to the SPF unit, newborns are placed in a synchronized litter of a foster mother. The other technique consists of transferring carefully washed three-day-old embryos into an SPF foster mother.

The aim of this study was to use embryo transfer for recovering the valuable genetic material of an infected herd after restarting with SPF rabbits of a lower genetic potential. Thus, the SPF health status of the rabbitry was to be recovered without loss of the genetic potential. This was achieved by performing superovulation and mating of contaminated does in order to recover day-3-embryos which were subsequently transferred to SPF recipient females.

MATERIAL AND METHODS

Superovulation of does

The genetically most valuable multiparous does from the New Zealand White type race "Cunistar" were selected. The rabbits (n=32) received ovarian stimulation according to the protocol described previously by Joly (1996). Each donor rabbit was treated with a total dose of 2 IU of pFSH (Stimufol, Merial, Brussels, Belgium) administered in five IM injections (respectively 0.25; 0.25; 0.625; 0.625 and 0.25 IU). The injections were administered every 12 h. Twelve hours after the last pFSH injection, ovulation was induced by an IM injection of buserelin (0.3 ml of Receptal, Hœchst, Frankfurt, Germany). Immediately afterwards, the does were naturally mated with "Cunistar" bucks with proven fertility.

Collection, selection and washes of embryos

Euthanasia was performed humanely by an IV injection of T61 (Hœchst, Frankfurt, Germany). Stimulated females were sacrificed 62 to 70 hours post-mating to recover compact morulae. After laparotomy, uterine horns were excised and dissected. Retrograde flushing of uterine horns was performed with DPBS (D-4031: Sigma, StLouis, USA) with 10% fetal calf serum (FCS) (Sigma, F-4135). After collection, the quality of the embryos was evaluated to select only embryos suitable for cryopreservation and further transfer. Only zona pellucida-intact embryos were washed. All embryos presenting visible cellular inclusions inside their mucin coat were discarded. Selected embryos from each donor were washed ten times to dilute contaminants as much as possible. All manipulations were performed following sanitary precautions described by Stringfellow and Seidel (1990) for bovine embryos.

Embryo freezing and thawing

The embryos were frozen in 1.5 M DMSO (Sigma, D-2650) in DPBS added with 20% FCS (Techakumphu and Heyman, 1987) in 0.15 ml transparent straws (CryobioSystem, Paris, France). The embryos were placed in a small column (± 10 mm) of 1.5 M DMSO separated from the rest of the straws by two air bubbles. The rest of the straws were completely filled with the same cryoprotectant. The straws were sealed with different colored caps, labeled and then placed immediately in the cooling machine (Embryofreeze, Biotronics, Leominster, UK). The embryos were cooled from 20°C to -7°C at 2°C/min. After a holding period of 5 min, the straws were seeded. After a second holding period of 5 min., the embryos were cooled to -35°C at a rate of 0.5°C/min. and then plunged directly into liquid nitrogen.

The embryos were thawed rapidly by immersing the straws for 20 sec in a water bath kept at 25°C. After each straw was emptied into an empty petri dish, the cryoprotectant was removed from the embryo by three step-wise dilution treatments for 5, 5 and 10 minutes in 1.0 M, 0.5 M, and finally no DMSO in DPBS with 20% FCS. After selection, only good quality embryos were transferred.

Embryo transfers

All surgical embryo transfers were performed under SPF conditions. The recipient rabbits (n=32) received IV injections of 75 IU hCG (Chorulon, Inter-

vet, The Netherlands) to induce pseudopregnancy. At 60h after injection the does were anesthetized with 5mg/kg of xylazine (Rompun 2%, Bayer, Germany) and 35 mg/kg of ketamine (Ketalar, Parke-Davis, USA). Only recipients with well-developed *corpora lutea* were selected for embryo transfer. The embryos were introduced into the fallopian tube via the *ostium abdominale* with a silicon catheter connected to a 1 ml syringe. Five to 9 morulae were transferred into each oviduct. After transfer, the foster mothers were caged individually until birth.

Health screenings were performed on sanitized rabbits 24-26 weeks after birth. Blood samples were sent to Dr. van der Logt in Nijmegen (ICLAS virus reference center). Ten of the 104 animals were screened according to FELASA guidelines.

RESULTS

Among the 32 stimulated does, one did not react and two were not fertilized. In total, 893 embryos were collected, among which 821 (92%) had an intact zona pellucida and were selected for cryopreservation. This corresponded to an average of 25.7 embryos per doe (821/32).

In order to conserve the genetic potential of the "Cunistar", 40 % of the frozen embryos were kept in liquid nitrogen (26 straws). Among the 36 straws thawed (478 embryos), 466 embryos were recovered (97.5 %), 417 of which were of good quality (89.5 %).

Ten to 18 embryos were transferred per recipient (mean = 13). After transfer, 25.4 % (106/417) of the embryos developed to term (Table 1). Two recipient does died before delivery, and the others gave birth to 106 pups. Two pups were stillborn. The mean number of live newborns per recipient was 3.47 (104/30).

Variations in percentages of newborns were observed among the donors (Table 2). Although the number of effective donors was low, it seems that the percentages of newborns per embryo transferred were influenced by the genotypes of the donors. Table 2 shows that group D did not produce any pups, while the number of recovered embryos was similar to the three other groups. On the other hand, group M had the best rate (38.4%) of newborns per transferred embryos.

Health screening performed on sanitized rabbits confirmed the recovery of the SPF health status of the rabbit breeding colony.

DISCUSSION

Restoration of the SPF condition was performed in this study by embryo transfer. This was preferred to aseptic hysterectomy because of possible transplacental disease contamination. For *Encephalitozoon cuniculi*, there is no consensus concerning transplacental transmission. Hunt *et al.* (1972) have shown this to occur both in experimentally and in spontaneously diseased rabbits. On the other hand, Wilson (1986) demonstrated the absence of vertical transmission. Since it is difficult during hysterectomy to avoid any contact between maternal and newborn blood which can also result in disease transmission, we preferred to apply embryo transfer.

Infectious agents do not cross the zona pellucida (Chen and Wrathall, 1989). Thus all embryos with a damaged zona pellucida were discarded. Ten washes were performed in order to obtain an extreme dilution of pathogens. Washing of the embryos is considered to be the best way to eliminate pathogenic agents from embryos (Thibier, 1990; Le Tallec *et al.*, 2001). IETS recommends using trypsin with the washes to eliminate pathogens that could adhere to the zona pellucida (Stringfellow and Seidel, 1998). A mucin coat surrounds the zona pellucida in rabbit species, however. Mucin is already present within a few hours after fertilization and its presence is critical for implantation (Murakami and Imai, 1996). Some pathogenic agents or contaminated cells may be present on the zona pellucida and covered by mucin, or inside the mucin coat. Their elimination by trypsin digestion is difficult to apply without damaging the zona pellucida, which starts softening even before complete digestion of mucin. Under these conditions, trypsin treatment cannot been used in rabbits. It is for this reason that all embryos presenting cellular inclusions between the zona pellucida and the mucin coat or inside the mucin coat were discarded.

All selected embryos were cryopreserved. This is the only technique to associate a stamping out under good conditions and a perfect synchronization between donors and recipients. Moreover, frozen embryos can be stored for further transfers. Different stages of embryo development can be frozen. Collection of day 3 embryos was chosen because morulae present the best resistance to cryopreservation (Bank and Maurer, 1974; Tsunoda and Sugié, 1977). Zygote collection can be another way of working in order to reduce the risks of disease transmission via the mucin coat. At the zygote stage, the mucin layer is already present, although it is thinner than in later stages of de-

Table 1. Number of pups born after transfer.

Donor	Recipient	Number of embryos transferred	Number of newborns	Number of stillbirths
M314	105	13	2	0
M314	112	14	/ (died)	/
M344	115	10	4	1
M344	117	11	3	0
M345	119	14	10	0
M345	152	13	5	0
M345	157	14	7	0
D232	165	14	0	0
D232	175	15	0	0
D232	176	15	0	0
D255	179	14	0	0
D255	203	15	0	0
C545	533	12	3	0
C545	209	12	0	0
C556	616	14	9	0
C556	569	14	5	0
M332	212	15	5	0
M332	428	16	10	0
M309	523	12	0	0
A416	166	15	0	0
A416	223	14	6	0
A416	275	14	8	0
A429	530	18	0	0
A429	565	17	0	1
284A430	609	14	8	0
A430	612	14	/ (died)	/
A425	614	18	5	0
C659	629	12	7	0
C659	630	12	2	0
C636	646	12	0	0
C636	656	11	3	0
C638	681	11	2	0
n = 17	**n = 38**	**n = 417**	**n = 104**	**n = 2**

Table 2. Results as a function of the genotype of the donors.

Donor	Total number of transferred embryos	Total number of newborns	%
A416	43	14	32.6
A425	18	5	27.8
A429	35	0	0.0
A430	28	8	28.6
Total of A	**124**	**27** [a]	**21.8**
C545	24	3	12.5
C556	28	14	50.0
C636	23	3	13.0
C638	11	2	18.2
C659	24	9	37.5
Total of C	**110**	**31** [a c]	**28.2**
D232	44	0	0.0
D255	29	0	0.0
Total of D	**73**	**0** [b]	**0.0**
M309	12	0	0.0
M314	27	2	7.4
M332	31	15	48.4
M344	21	7	31.8
M345	41	22	53.7
Total of M	132	46 [c]	34.8
Mean	**417**	**104**	**24.9**

Values with different superscripts are significantly different (Chi-Square: $p < 0.05$).

velopment. Nevertheless, this procedure may reduce the number of pups after transfer. This is caused by the fact that selection of good embryos is less efficient. Under our conditions, nearly 90% of the embryos recovered after freezing were transferable, and grossly 25% of them developed to living newborns after transfer (see Table 1).

Table 2 shows variations in percentages of newborns among donors. This was previously observed by Vicente and Garcia-Ximenez (1993) and Joly (1997). They described variations in embryo survival after cryopreservation as a function of genetic origin. This could be due to different uterine environments, which influence fertilization and development rates (Torrès *et al*, 1986).

In conclusion, we have demonstrated that the transfer of washed and cryopreserved embryos with mucin coat is an effective method for eliminating pathogenic agents in rabbit breeding units.

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