CRYOPRESERVATION OF GENETIC RESOURCES IN RABBIT SPECIES : PRACTICAL APPLICATION

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Abstract - In this paper, methods using frozen germplasm were considered to preserve ex situ the genetic resources in rabbit species. After thawing of frozen semen and embryos, the mean values of fertile offsprings (= pups borned from insemination or transfer that became fertile) were set to 2~3 per frozen semen dose and 0.3 per frozen thawed embryos. For any rabbit populations, we estimate that a total number of 400 semen doses and 500 embryos need to be frozen to supply enough fertile offsprings. Despite the impacts of the variability in efficiency of establishing a bank of semen and embryos, our results demonstrate the effectiveness of experiment proceedings for the long term storage of valuable rabbit germplasm.

INTRODUCTION

The rabbit (*Oryctolagus cuniculus)* is widely spread in the world; it exists in the form of a vast array of breeds, synthetic lines, strains. Many genotypes present a specific value and interest for scientific, economic and sociocultural aspects. Several of these populations are today threatened with extinction as a consequence of the very limited number of genotypes exploited today in intensive animal production, but also because of the fact that many of the local breeds are no longer supported by traditional backyard farming (BOLET *et al.*, 1996). While there is still today a need for evaluation of rabbit breed resources from living animals, it is also necessary to raise measures for safe conditions of preservation of this genetic diversity based on the conservation of frozen material that will allow to recreate a population. This approach, which must be considered as a complement to strategies of *in situ* conservation is particularly well adapted to the rabbit (JOLY *et al.*, 1994). In that species, efficient freezing procedures has been previously described for 2 types of germplasm cells: spermatozoa and embryos. The artificial insemination with frozen semen can be used on field and the technics of embryo freezing provide results similar to those obtained after transfer of fresh embryos (RENARD *et al.*, 1984).

The aim of this paper is to precise the conditions of semen and embryo banks implementation to preserve the genetic resources in rabbit species. So, we will first provide data on the efficiency of preservation techniques, based on trials performed the past 2 years within the frame of a concerted action (DG XII, PL 920184) between groups belonging to 3 European countries. Then, we will use the results to estimate the number of semen doses and embryos that need to be frozen for any rabbit breeds.

SEMEN PRESERVATION

Technical aspects

Classical methods using natural stimulation of neurosensorial mechanisms was used to produce semen. Fresh ejaculates were recovered with an artificial vagina and sperm was examinated under light microscopy immediately after collection (BOUSSIT, 1989). Only ejaculates with sufficient sperm motility (70%) were kept. Sperm of bucks without sexual behaviour can be collected by other methods: electroejaculation (BADURA,

1976) or surgery to remove epididymal sperm. These artificial methods of sperm collection are not easy to apply on a large scale and must be reserved for males with high genetic value (mainly lines which have a biomedical interest).
Among the different technics available to freeze semen, we choose two common procedures to be used routinely: COUROT (1977) and WEITZE (1979). Both procedures imply a final dilution of sperm at 1/5~1/10 in two steps to ensure a better preservation: 1) predilution at ambient temperature/35°C in a basis Medium (Tris/citric acid/Glucose/Egg Yolk) added to DMSO and equilibration at 5°C during 1 hour; 2) final dilution in Basis Medium/Lactose added to Glycerol, portioning in straws of 0.5 cc and freezing in LN2 vapour (final concentration: DMSO 4.5~5%, Glycerol 1~1.6%). The straws were stored in LN2 at -196°C and thawed in a water bath at 37°C. Sperm fertilizing ability was determined by studying the fertility of does inseminated with a single semen dose of 0.5 cc into vagina (THEAU-CLEMENT and ROUSTAN, 1991) or by endoscopic way (transcutaneous insertion of a needle and punction of uterine horns).

Efficiency of semen cryostorage

Semen cryostorage efficiency is provided by the results realized in experimental stations. These results are based on two situations: one related with a commercial line (HYplus) selected for production traits and the other related with transgenic lines developped in small populations for their potential biomedical interests. For these 2 types of populations, more than 50 bucks were sampled and collected. Frozen semen was subsequently thawed and used to inseminate about 650 females (respectively 525 and 129). Similar work was performed at a lesser scale with different endangered breeds of rabbit raised by fancy breeders. Table 1 gives an estimation of the efficiency of semen cryostorage procedures extrapolated from our works. Efficiency is defined from the number of fertile young rabbits that can be produced from one dose of frozen semen.

Table 1: Efficiency of production and freezing of semen

Semen production			Semen viability after thawing		
% of donor buck (a)	nb of doses per ejaculate	frequency of collection	Fertility rate	Pups per delivery	Fertile offsprings per frozen dose
50%	12 [6~23]	2 ~ 4 /week	50% [46~100%]	7 [4.3~11.5]	2,5

(a) donor buck = buck with frozen semen collected by artificial vagina

The percentage of donor male capable of providing sperm that we obtained in our work (50%) is similar to data previously reported (VALENTINI *et al*, 1988). From the ejaculates, only about one third appears to be suitable for freezing in relation with their motility. Even if the final dilution is low, each ejaculate can yield on average 12 frozen doses. From some males only 6 doses could be frozen per ejaculate, while about 15% were able of providing more than 20 doses. This estimate fits with our experience on bucks collected from endangered breeds in collaboration with the fancy breeders association. However, since a low number of such breeds have been collected at the moment, we cannot exclude that a greater variability could be encountered.
We estimated on average 50% the fertility rate of inseminated does. In our work, higher values were obtained with a commercial line selected on litter size (66.5%) and with several transgenic lines (79.8%). This is mainly due to the fact that commercial line was selected on reproduction performances and the transgenic lines were inseminated by endoscopic way. Since this technique can not be performed systematically, we consider that the 50% is a more representative result compatible with routine application of insemination in farm condition (FARGEAS, 1995; CASTELLINI *et al.*, 1988).
Number of pups delivered per pregnancy varied between 4.3 and 11.5 with a mean value of 8.3. This mean value is higher than the data obtained from the breeders of various breeds, which made us consider 7 pups as a representative value of the number of young rabbits that it is possible to obtain after each artificial insemination. From these youngs, about 70% became fertile which thus resulted in a mean number of fertile offsprings of 2 to 3 per frozen dose.
Overall, from 100 frozen semen straws stored in a cryobank, we can predict to get approximately 250 fertile young rabbits after insemination of recipient does.

EMBRYOS PRESERVATION

Technical aspects

The production of frozen embryos were routinely achieved by 2 procedures: 1) superovulation and 'slow freezing'; 2) non stimulation and vitrification.

1. Superovulation and "slow freezing": A follicle stimulating hormone obtained from porcine pituitaries (FSH) was used to overstimulate the rabbit ovaries. Each female was treated with a total dose of 2 mg FSH (Stimufol, Rhône Merieux, France) that has been administered in a subcutaneous way with 5 injections (twice a day; respectively 0.25; 0.25; 0.625; 0.625 and 0.25 mg). Eight hours after the last injection, females were inseminated or naturally mated with bucks of proven fertility. Immediately after, the ovulation was induced with an intramuscularly injection of a GnRH analogous (0.2 ml of busereline; Receptal, Distrivet, France). The main advantage of the FSH treatment is that all does independently of their receptive status can be synchronized for the same experiment of collection.
   The "slow freezing" procedure is a progressive dehydratation of the embryos, by substitution of a part of intracellular water by a cryoprotectant solution to avoid any formation of ice crystals that can cause injuries on the intracellular membranes. It involves the use of a programmable freezer (Cryocell 1200, IMV, L'Aigle, France), a portable equipment which is very easy to use in the field. Embryos were plunged during 5 minutes each time in 3 successives baths of PBS1 containing respectively 0.5 M, 1.0 M and 1.5 M DMSO. They were introduced into preidentified straws (0.25 ml), placed into the programmable freezer and seeded after 5 minutes at -7°C. Embryos were cooled until -30°C at a rate of 0.5°C per minute. Then, the straws were directly plunged and stored into liquid nitrogen at -196°C.

2. Non stimulation and vitrification : Only receptive multiparous does were mated with fertile bucks. After mating, the females were injected by intramusculary way with 25 IU hCG (Coriogan, Ovejero laboratories, Leon, Spain). This method of embryo production is less costly than the FSH treatment. However, the synchronization of females in a single batch for experiments is more difficult because only does in a receptive status can be used.
   Recently a new technique has been developed and yielded a viable alternative to the slow freezing procedure. Vitrification is the solidification of a liquid brought about by an extreme elevation in viscosity during cooling. Highly concentrated aqueous solutions of cryoprotectants are able to supercool to very low temperatures. They become so viscous that they solidify without the formation of ice. Successful vitrification requires the use of a highly concentrated yet effectively non-toxic solution of cryoprotectants and high cooling and warming rates.
   The vitrification procedure previously described by VICENTE and GARCIA-XIMENEZ (1994, 1996) was carried out in 2 steps. First, embryos were placed during 2 minutes in a PBS1 medium containing 12.5% Ethylen Glycol (EG) and 12.5% DMSO. In the second step, embryos were suspended in the final vitrification solution (20% EG and 20% DMSO in PBS1), loaded into 0.25 ml plastic straws and then plunged directly into liquid nitrogen. Embryos were exposed to the final vitrification solution for a total of 1 minute.
   For both procedures, embryos were recovered *post mortem* at compacted morula/blastocyst stage 65 to 72 hours after induction of ovulation by perfusing the genital tract with a phosphate buffer solution supplemented with 20% heat inactivated serum (PBS1).The main inconvenience is that it implies killing the females. It must be notified that it is possible to collect *in vivo* females several times (embryos are recovered in the vagina with a cannula), but the practical applications are more difficult and must be reserved only for females with a high genetic value (GARNIER *et al*, 1988). Only morulae and blastocysts of excellent and good quality according to morphological criteria (HAFEZ, 1993) were frozen.
   The thawing of embryos must be realized as rapidly as possible to avoid the recrystallization of intracellular water. Straws were plunged directly into a water bath at 20°C. Cryoprotectant was removed according to freezing procedures: slow freezing embryos were placed during 5 minutes each time in 2 successives baths of PBS1 containing respectively 1.0 M and 0.5 M DMSO, and vitrified embryos in two successive baths of 0.3 M sucrose in PBS1 and PBS1.
   The embryo viability after thawing was assessed by *in vivo* development. The embryos (n=8-12 embryos/recipient) were transferred into the oviduct/uterine horns of pseudo-pregnant recipients, 65h-72 h after induction of ovulation. Now, non surgical technics have been simplified considerably by the development of endoscopic methods for embryo transfer (GARCIA-XIMENEZ *et al.*, 1991; BESENFELDER and BREM, 1993).

Efficiency of embryo cryostorage

The embryo cryostorage efficiency is provided by results from on-field trials realized in experimental stations and directly on farms. These results are based on two situations: "FSH/slow freezing" related with commercial lines, endangered breeds, strains with biomedical interests,... and "no stimulation /vitrification" related with commercial lines selected on breeding performances.
Overall, about 1200 does (respectively, 300 and 886) were sampled and collected. Frozen embryos were subsequently thawed and transferred into approximately 300 recipient females (respectively 144 and 153). Table 2 gives an estimation of the efficiency of the embryo cryostorage procedures extrapolated from our works. Efficiency is defined from the number of fertile young rabbits that can be produced from one frozen embryo.

Table 2: Efficiency of embryo production and viability after thawing

Embryo production			Embryo viability after thawing	
Type of procedure	% of donor does (a)	Frozen embryos / donor	Number of pups / transferred embryo	Fertile offsprings / frozen embryo
FSH + 'slow freezing'	70% [65~90%]	18 [10.9~30.4]	0.44 [0.23~0.51]	~ 0.3
no stimulation + vitrification	80% [62~84%]	9 [7.5~9.5]	0.47 [0.39~0.52]	~ 0.3

(a) donor does = does with frozen embryos

Whatever the treatment of embryo production (FSH or "no stimulation"), approximately 70%~80% of the collected females have yielded freezable embryos [range:65% to 90%]. The mean number of freezable embryos collected per donor were 17.7 [range:10.9~30.4] for the FSH protocol and 9.2 [range:7.5~9.5] for the "no stimulation" protocol. These data are classically variable according genotypes and environmental conditions of production. Nevertheless, the superovulation protocol can be used to enhance the production of freezable embryos per donor doe and to provide about 2 straws of frozen embryos per donor.
Overall, 85-95% of the frozen embryos (slow frozen and vitrified) have been judged morphologically viable after thawing. After transfer of these embryos, approximately 80% of the recipients established a pregnancy and littered (range according recipient genotype = 59-92%). The embryo survival rate (number of pups/ frozen embryos transferred into delivering recipient does) was on average 54% and comparable to the *in vitro* development after 24-48 hours of culture in incubator (62%). The mean value of pups per frozen-thawed embryos was 0.44 for "slow freezing" and 0.47 for vitrification. Thus, in the range from 0.23 to 0.52, almost a quarter to half of the frozen embryos produced pups. From these youngs, about 70% were became fertile which thus resulted in a mean number of fertile offsprings of 0.3 per frozen embryo, for both protocols that can be used routinely for embryo cryobanking.
Overall, from 100 frozen embryos stored in cryobank, we can predict to get approximately 30 fertile young rabbits after the transfer into recipient does.

PRACTICAL APPLICATION TO CRYOBANKING

It is currently considered that a given breed can be reconstituted from a starting group of 25 unrelated males and 25 unrelated females donors (SMITH, 1984).
In the case of sperm conservation, the objective is to ensure for each donor male a minimum of 40 fertile offsprings. This objective corresponds to the extreme situation where only a few animals remained of this population which could be only reconstituted through successive backcrossings.
In the case of embryos conservation, the objective is to obtain 6 offsprings, this is to be sure to obtain at least one male and one female breeder. Moreover, if there are no more alive animals in a given breed, any population can be directly reconstituted at the first generation after the transfer of frozen embryos.
Table 3 gives an estimation of the samples stored according objectives of conservation and the efficiency of freezing procedures.

Table 3: Germplasm preservation: definition of the sample to be stored per genotype

TYPE OF	Objectives	Efficiency	Sample to be stored		
GERMPLASM	Total pups (/Donor)	Fertile pups / Dose	Total doses /genotype	Number of doses/Donor	Number of treated animals
Semen	1000 (40)	2.5	400	16	50
Embryo (a)	150 (6)	3	50 (~500 embryos)	2 (16~24 embryos)	75 (no stimulated) 50 (superovulated)

(a) dose=straw containing 10 frozen embryos (8~12) currently used to transfer 1 recipient doe

Conserving the same objectives of conservation, we propose for rabbit species:

- In the case of semen preservation, the objective is to obtain 1000 pups (40 offsprings x 25 donor bucks) after artificial insemination with frozen semen. The total number of frozen dose to be stored per genotype was set to 400 (1000 pups / efficiency 2.5). 50 bucks must be sampled and collected to obtain frozen semen from 25 donor bucks (at least 16 doses/donor).
- In the case of embryo preservation, the objective is to obtain 150 pups (6 offsprings x 25 donor does) after embryo transfer of frozen embryos. The total number of frozen embryo to be stored per genotype was set to 500 (150 pups / efficiency 0.3), *i.e.* about 50 straws containing 8~12 embryos (2 straws per donor doe). According to the protocol of embryo production (% of donor females and number of freezable embryo per donor), 62 non stimulated does or 36 superovulated does must be sampled and collected to obtain frozen embryos from 25 donor does. To take into account the variability of individual response of the does to embryo production treatment, we consider a sample of 75 non stimulated does or 50 superovulated does and a total of 500 frozen embryos per genotype as a good margin of security.

These values are specific to each specie according to the physiological parameters (fertility rate, prolificity, litter size,...) and the efficiency of the freezing procedures. For example in cattle species, the minimal sample to be stored per genotype was set to 2000 doses of frozen semen and 300 frozen embryos (OLLIVIER and RENARD, 1995). To define sampling strategies, more precise methods related to the objectives of conservation will be published later.

CONCLUSION

Despite the impacts of the variability on efficiency of establishing banks of semen and embryos, our results demonstrate the effectiveness of procedures for the long term storage of valuable rabbit germplasm. In this paper, we give an overview of results which are to be published in more detail at a later date where the variability of individual response to the protocols of germplasm production and the objectives of conservation will be taken into account. The total number of semen doses and embryos that need to be stored per genotype is a key aspect for further estimation of the cryostorage costs.

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Cryopréservation des ressources génétiques dans l'espèce du lapin : applications pratiques - Dans cet article, les méthodes de conservation des cellules germinales ont été évaluées pour conserver *ex situ* les ressources génétiques chez le lapin. Après décongélation de la semence et des embryons, le nombre moyen de descendants fertiles (= lapereaux issus d'insémination artificielle ou de transfert embryonnaire qui deviendront fertiles) est de 2 à 3 par dose de semence et de 0.3 par embryon congelé. Au total, nous estimons que 400 doses de semence et 500 embryons doivent être congelés pour conserver une population de lapin. Malgré les inconvénients de la variabilité des résultats de production sur la mise en place d'une cryobanque, nos résultats démontrent l'efficacité des protocoles de congélation utilisés pour la conservation à long terme des ressources génétiques sous forme d'embryons et de semence.

Criopreservación de recursos genéticos en el conejo : aplicacion práctica - En el presente trabajo, se evalúa la posible utilización de los procedimientos actuales de congelación de germoplasma para conservar *ex situ*, de manera eficiente recursos genéticos en la especie cunicola. El número medio de descendientes viables obtenidos por inseminación con semen congelado es de 2 a 3 gapazos. Cuando se transfieren embriones criopreservados, la tasa de supervivencia sobre embrión congelado es del 30%. Se estima que se requieren congelar 400 dosis de semen y 500 embriones para conservar una población de conejo. Pese a los inconvenientes que la variabilidad de los resultados obtenidos presenta sobre la constitución de un banco, los resultados demuestran la eficacia de los protocolos de congelación utilizados para la conservación a largo plazo de los recursos genéticos en forma de embriones y de semen.