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Original article

A sucrose-DMSO extender for freezing rabbit semen

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Summary — The aim of this study was to define a simple extender for freezing rabbit semen from selected males used to inseminate selected does to obtain embryos for an embryo bank. Four experiments were carried out. In the first experiment, freezing extender was defined on the basis of the results of the post-thawing motility rate. Three factors and their interactions were studied: final concentration of dimethyl sulphoxide (DMSO) (1, 1.25, 1.5 and 1.75 M), egg yolk (0% of 10 v/v) and sugar (none, or 0.5 M of glucose, lactose, sucrose, or maltose). The sucrose and 1.75 DMSO improved significantly the post-thawing motility rate (sucrose 1.75 M DMSO extender: 42 ± 3%). In the second experiment, this freezing extender was used to freeze semen from three lines. The post-thawing motility and normal acrosome rates were similar among the lines when the covariates, fresh motility and normal acrosome rates, respectively, were used in the analysis (52 ± 1 and 66 ± 1%, respectively). In the third experiment, frozen semen from the White New Zealand line (NZ) was tested by morphological normality and viability of embryos recovered. Recovery data from NZ does inseminated with fresh and frozen semen were compared. No significant differences were found in the number of normal embryos obtained per donor doe (8.9 ± 0.5) and in their survival after vitrification (52% of live foetuses at 29 days of gestation). The sucrose-DMSO extender and freezing procedure used in this work can offer satisfactory results to apply in conservation and genetic programmes.

semen / freezing / sucrose / rabbit

Résumé — Un milieu saccharose-DMSO pour la congélation de la semence de lapin. *L'objectif de notre étude a été de définir un milieu pour la congélation de la semence de lapin, afin d'obtenir, après insémination artificielle, des embryons dans le cadre de la constitution d'une banque d'embryons. Quatre expériences ont été pratiquées. Dans la première, le milieu de congélation a été défini en fonction de la motilité postdécongélation. Trois facteurs et leurs interactions ont été étudiés : concentration finale de DMSO (1, 1,25, 1,5, 1,75 M), jaune d'œuf (0 ou 10 %) et sucre (aucune, ou 0,5 M de glucose, lactose, saccharose ou maltose). Le saccharose et le DMSO 1,75 M améliorent significativement le taux de motilité postdécongélation (42 ± 3 %). Dans la deuxième expérience ce milieu a été utilisé pour congeler la semence de trois souches de mâles. Aucune différence significative n'a été observée entre les souches pour le taux de motilité et le pourcentage d'acrosomes normaux (respectivement, 52 ± 1 et 66 ± 1 %). Dans la troisième expérience, la population des embryons obtenus et leur viabilité ont été comparées sur un lot de lapines White New Zealand inséminées avec de la semence fraîche et*

congelée. Le nombre d'embryons normaux par femelle (8,9± 0,5) et leur viabilité après vitrification (5 2 % à 29 jours de gestation) ne varient pas significativement entre les groupes. Le milieu saccharose-DMSO et le protocole de congélation utilisé dans ce travail peuvent offrir des résultats satisfaisants pour une application à des programmes de conservation des ressources génétiques.

semence / congélation / saccharose / lapin

INTRODUCTION

Artificial insemination in rabbits is usually performed with fresh or recently stored sperm. This is because of the generally unsatisfactory results with frozen semen (Stranzinger et al, 1971; Maurer et al, 1976; Hanada and Nagase, 1980; Constantini, 1989; Fargeas, 1995). In order to better utilize semen from males of high genetic value, for production as well as for conservation and genetic programmes, use of frozen semen is important.

In general, rabbit semen freezing medium contains egg yolk and dimethyl sulphoxide (DMSO) or acetamide as a cryoprotectant (Stranzinger et al, 1971; Hanada and Nagase, 1980; Chen et al, 1989). Generally, 1 to 1.5 M of DMSO has been used in the extender to obtain 40–50% motility and 30–70% normal acrosomes (Weitze et al, 1976; Castellini et al, 1992; Martin, 1993). Using 1 M acetamide in the extender improves the normality acrosome rate (60–80%; Chen et al, 1989). Both the DMSO extender and the acetamide extender are used with similar freezing procedures: samples are cooled for 4–5 h at 5 °C before freezing in vapour nitrogen. Recently, Chen and Foote (1994), using a programmable freezer, demonstrated that mechanical seeding of extended semen at −6 °C substantially improved post-thaw progressive motility of spermatozoa (60%).

The aim of this study was to define a simple extender for freezing rabbit semen from selected males used to inseminate selected does to produce embryos.

MATERIALS AND METHODS

Experimental animals

Mature male rabbits from three genetic lines were used in this study. Two lines were selected on the basis of litter size at weaning: Synthetic (SY) and White New Zealand (NZ). The third line (SB) was selected on the basis of growth rate from weaning to slaughter (28–63 days of age).

Nonlactating multiparous does (4th or 5th parity) belonging to NZ and SY lines were used for insemination and embryo transfer, respectively. All rabbits were kept individually under the same environmental conditions (16 h light: 8 h dark, 20–25 °C).

Semen collection and evaluation

Two ejaculates were recovered per male and week. Semen was collected using an artificial vagina. The following measurements were taken from fresh semen:

– Volume: measured in a graduated conical tube.

– Percentage of motile sperm: a semen sample diluted 1:100 in Tris-citrate was evaluated subjectively by examining spermatozoa on a glass slide at 37 °C. Two experienced evaluators determined the progressive motile spermatozoa into three to five microscopic fields from each ejaculate.

– Concentration per milliliter: measured with a hemocytometer.

– Normal acrosome: the proportion of sperm with normal intact acrosome was estimated using interference contrast optics at a magnification of x1250. Spermatozoa were fixed with glutaraldehyde 2% in phosphate-buffered saline (PBS) (Pursel and Johnson, 1974).

If the concentration of spermatozoa exceeded 200×10^6 mL^{-1}, it was extended to 120×10^6 mL^{-1} with Tris-citrate.

Examination of frozen-thawed semen for the percentage of progressively motile spermatozoa was conducted in the same way as for fresh semen.

Freezing procedure of sperm

The semen and extender were mixed at room temperature (25 °C) in equal volumes. The extended semen was packaged in 0.25 mL plastic straws. These straws were then placed at 5 °C for 120 min. After cooling, the straws were placed at –30 °C for 15 min in a freezer before being plunged in liquid nitrogen. Thawing was carried out by immersing the straws in a water bath at 40 °C for 15 s.

Insemination procedure

Receptive does were inseminated with 20 x 10^6 motile spermatozoa in 0.5 mL of Tris-citrate medium or 0.5 mL of freezing medium for fresh and frozen semen, respectively. Receptivity of does was checked by vulva colour. Insemination was carried out with a curve glass pipette (0.5 mm diameter). Does were injected intramuscularly with 0.2 mL gonadorelin (Fertagyl, Intervet) at the time of insemination. All inseminated does were nonlactating multiparous females (4th or 5th parity).

Recovery embryos

Morulae were recovered postmortem 64–66 h postcoitum by perfusing each oviduct and one-fourth of each uterine horn with 5 mL of Dulbecco's phosphate-buffered saline (PBS, Sigma) supplemented with 20% heat-inactivated rabbit serum (PBS + 20%). Embryos were scored according to morphological criteria before freezing (Hafez, 1993).

Vitrification procedure

The vitrification consisted of two steps. In the first step, normal embryos were pipetted into 0.2 mL of PBS1 medium and placed in a plastic culture dish. Then 0.2 mL of a solution consisting of 25% (v/v) EG plus 25% DMSO (v/v) in PBS1 was added and diluted by agitation. The embryos were left for 2 min in this medium. In the second step, 0.6 mL of the same initial cryoprotective solution (25/25, EG/DMSO) were added and diluted quickly. Embryos suspended in the final vitrification solution were loaded into a 0.25 mL plastic straw (IMV, L'Aigle, France) and then plunged directly into liquid nitrogen. The two vitrification steps were carried out at 20 °C and the final vitrification solution contained 20% (v/v) EG and 20% (v/v) DMSO in PBS1 medium.

The straws contained three fractions separated by air bubbles: the first fraction consisted of PBS1 in a cotton plug, the second fraction contained the embryos suspended in vitrification medium (0.1 mL) and the third fraction consisted of the PBS1 medium. Straws were sealed with coloured plastic. The total duration of embryo exposure to the final vitrification solution before being plunged into the liquid nitrogen was 1 min. Each straw contained seven embryos.

Devitrification was carried out in a water bath at 20 °C, then the fraction with the embryos (second) was poured and mixed into a culture dish containing 0.8 mL of 0.33 M sucrose in PBS1 medium. After 5 min, the embryos were washed twice in Ham's F10 and supplemented with 20% of homologous rabbit serum or fresh PBS1 before culture or oviductal transfer, respectively.

Embryo culture

After devitrification, morulae were morphologically examined, and only normal embryos in Ham's F10 were cultured in a humidified atmosphere of 7% CO_2 in air at 39 °C for 48 h (Vicente and García-Ximénez, 1994).

Embryo transfer

Recipient does were induced to ovulate 60 h before transfer with an intravenous dose of 25 IU hCG in accordance with Vicente and García-Ximénez (1994). All the recipient does were multiparous females (4th or 5th parity). The recipients were anaesthetized by injecting a 5:1 ketamine chlorohydrate:promethazine solution at

the rate of 1.2 mL kg^{-1} of body weight. After vitrification, transfers were carried out by ventral midline laparotomy and the embryos were transferred into the oviducts of the recipients.

Experiment 1: Effect of sugar, DMSO and egg yolk in the extender medium on post-thawing motility

Eighteen White New Zealand males belonging to a selected line by litter size were used in this experiment. Forty freezing media were studied for their effects on post-thawing sperm motility. Freezing extender consisted of 0.1 M sugar and 2 to 3.5 M of DMSO in Tris-citrate (0.25 M trishydroxymethylaminomethane, 83 mM citric acid) and with or without 20% of egg yolk. Penicillin (1 000 U mL^{-1}) and streptomycin (1 000 μg mL^{-1}) were added. Three different pools of six ejaculates were used (18 males). Each pool was split and extended with all media. Three replicates for the freezing medium and two straws for each replicate were evaluated. The procedures for extensing and freezing-thawing have been described earlier.

A factorial analysis of variance after arcsin transformation of post-thaw sperm motility data was used to evaluate the main effects of sugar, concentration of DMSO and egg yolk in the freezing medium and its interactions. A protected least significant difference (LSD) test was used to compare means.

Experiment 2: Effect of genetic lines of males on quality of fresh and frozen semen

The results of experiment 1 showed that the addition of sucrose and 3.5 M DMSO improved sperm motility. The extender in this second experiment was 0.1 M sucrose and 3.5 M DMSO in Tris-citrate. Extension, freezing and thawing procedures have been explained earlier.

Twenty-five ejaculates from 25 sexually adult males were used from each of three lines (NZ, SY and SB). The effect of the genetic line on the volume, concentration and sperm production was evaluated by analysis of variance. After arcsin transformation, the effect of the genetic line motility and normal acrosome rates from fresh and post-thawed semen were analysed by analysis of variance. In addition, the percentages of motile sperm and normal acrosome were analysed by covariance. The covariates were motility and normal acrosome rate in fresh semen, respectively. A protected LSD test was used to compare means.

Experiment 3: Effect of frozen semen on normal embryo recovery and postvitrification viability of recovered embryos

Fifty-two receptive does from the NZ line were inseminated with fresh or frozen semen of males from the same genetic line. The following parameters were recorded: ovulation rate, normal embryos, abnormal embryos and oocytes. Two nonovulated does were excluded from analysis.

Three hundred thirty-five normal embryos were vitrified (177 from donor does inseminated with fresh semen and 158 from donor does inseminated with frozen semen).

In vitro development: After devitrification, 119 normal embryos from fresh semen and 101 normal embryos from frozen semen were cultured for 48 h. Development to the expanded blastocyst stage was then recorded.

In vivo survival: After vitrification, 98 normal embryos of the NZ line were transferred to the SY recipient does. In a previous study, Vicente and García-Ximénez (1993) demonstrated a positive effect of SY recipients on the survival at birth. Seven transfers were performed and each recipient doe received seven embryos from donor does inseminated with fresh semen and in the the opposite oviduct seven embryos from donor does inseminated with frozen semen. Recipients were slaughtered on day 29 of gestation; each uterine horn was opened to record the number of live foetuses. Survival rate was evaluated as the percentage of live foetuses in relation to the number of transferred embryos.

An analysis of variance was used to evaluate the effect of frozen semen on the number of normal and abnormal embryos, and oocytes by donor doe. In vitro and in vivo survival rates were analysed by a chi-square test with Yate's correction.

RESULTS

Experiment 1: Effect of sugar, DMSO and egg yolk in the extender medium on post-thawing motility of sperm

Extending medium with egg yolk did not improve the percentage of motile sperm post-thaw for the two-step freezing method used in this work (24 ± 1 without egg yolk and 27 ± 1 with 10% v/v of egg yolk in final freezing medium). Thus, egg yolk is not needed in the freezing extender.

Significant differences were observed for sugar and for the level of DMSO in the percentage of motile sperm post-thaw (34% to 1.75 M vs 27, 20 and 20% to 1.5, 1.25 and 1 M of DMSO in the freezing medium, respectively, $P < 0.05$; table I). An extender without sugar gave the lowest results of post-thawing motility (17%; table I). The sucrose extender results were better (32%, $P < 0.05$; table I). Only another disaccharide, maltose, gave similar results (28%; table I). No interaction was found among the main factors; however, when the freezing medium contained 0.5 M of sucrose and 1.75 M of DMSO, the percentage of post-thaw motile sperm was 44%.

In this first experiment we have defined a simple sucrose-DMSO extender on the basis of the post-thawing motility.

Experiment 2: Effect of genetic lines on quality of fresh and frozen semen

Line SY showed greater sperm production than either NZ or SB (291 x 10^6 vs 221 x 10^6 and 141 x 10^6, $P < 0.05$; table II). These significant differences were due to different semen volumes and sperm concentration among the lines.

No differences were found in the percentage of motile sperm post-thaw among the lines with or without a covariate (52%). The covariate was the percentage of motility of the fresh semen; it was significant with a coefficient of 0.34 ± 0.11.

The percentages of normal acrosome were different among lines. The SB line showed the worst percentage (62 vs 70% of line SY, $P < 0.05$; table II). However, this difference disappeared when the covariate was used in the analysis (66 ± 1, coefficient: 0.70 ± 0.22, $P < 0.001$).

Table I. Experiment 1: Effect of sugar and concentration of DMSO in the freezing medium on the post-thaw percentage of motile sperm (least square means ± standard error).

Sugar	*No sugar*	*Glucose*	*Sucrose*	*Lactose*	*Maltose*
Motile sperm (%)	17 ± 2^a	24 ± 2^b	32 ± 2^c	25 ± 2^b	28 ± 2^{bc}
DMSO	*1 M*	*1.25 M*	*1.5 M*	*1.75 M*	
Motile sperm (%)	20 ± 2^a	20 ± 2^a	27 ± 2^b	34 ± 2^c	

^{abc} Values with different superscripts are statistically different ($P < 0.05$).

Table II. Experiment 2: Effect of genetic line of rabbit in the volume, concentration, motility, normal acrosomes (NAR) and abnormality sperm (Abn) (least square means ± standard error).

Line	Volume (mL)	Spz/mL (x10^6)	Semen production spz/ejaculate (x 10^6)	Fresh Motility (%)	Fresh NAR (%)	Fresh Abn (%)	Post-thaw Motility (%)	Post-thaw NAR (%)
SY	0.9 ± 0.1^a	340 ± 31^a	291 ± 29^a	80 ± 2^a	90 ± 1^a	1 ± 0.3^a	52 ± 2	70 ± 3^a
NZ	0.9 ± 0.1^a	262 ± 26^b	221 ± 25^b	81 ± 2^a	89 ± 1^a	1 ± 0.3^a	53 ± 2	64 ± 4^{ab}
SB	0.6 ± 0.1^b	233 ± 26^b	141 ± 25^c	73 ± 2^c	85 ± 1^b	3 ± 0.3^b	50 ± 2	62 ± 3^b

Spz/mL: number of spermatozoa per mL. ^{abc} Values with different superscripts are statistically different ($P < 0.05$).

Experiment 3: Effect of frozen semen on normal embryo recovery and postvitrification viability of recovered embryos

Forty-five donor does of 50 ovulating does produced normal embryos (23–91%) from doe groups inseminated with frozen semen and 22–87% from does inseminated with fresh semen. No differences were found in normal or abnormal embryos and successful recovery rate between the fresh and frozen semen (table III). The number of unfertilized oocytes differed between groups (2.3 vs 0.3, for frozen and fresh semen, respectively, $P < 0.05$; table III). The covariate, ovulation rate, was significant in the production of normal embryos and this coefficient was 1.06 ± 0.17. The percentage of normal embryos from total recovery (oocytes and embryos) was 76% for does inseminated with frozen semen and 86% from does inseminated with fresh semen.

Postdevitrification, 95% of the vitrified embryos scored as normal (318/335). A similar percentage of expanded blastocysts was obtained after 48 h in vitro culture of embryos from does inseminated with fresh and frozen semen (72/119 and 60/101, respectively, 60%).

All recipients became pregnant in vivo. The survival rate was 55% (27 live foetuses) and 50% (24 live foetuses) for transferred embryos from does inseminated with fresh and frozen semen, respectively. Over-

Table III. Experiment 3: Effect of frozen semen rm on embryo recovery (least square means ± standard error).

Group	Does	Ovulation rate	Normal embryos	Abnormal embryos	Oocytes	Successful recovery (%)
Frozen	25	12.3 ± 0.6	8.9 ± 0.8	0.5 ± 0.3	2.3 ± 0.5^a	91
Fresh	25	13.4 ± 0.5	8.9 ± 0.7	1.1 ± 0.3	0.3 ± 0.4^b	87

Does: number of ovulating donor does; successful recovery: donor does with normal embryos/ovulating donor does. ^{ab} Values with different superscripts are statistically different ($P < 0.05$).

all survival of vitrified embryos of this rabbit line was 52%.

DISCUSSION

A sucrose extender gave better results with bull semen and relatively fast freezing methods were observed to provide better protection by nonpermeating sugars (Nagase et al, 1968). Several authors have demonstrated better protection of sperm with relatively high levels of DMSO (0.8 to 1.2 M, Castellini et al, 1992; Martin, 1993). Post-thaw sperm motilities observed by these authors were 53 and 46%, respectively. In addition, Castellini et al (1992) showed that about 1.7 M DMSO provoked a negative effect on post-thaw sperm motility (23%). In the present study, better results on sperm motility were obtained with a final concentration of 1.75 M DMSO. Therefore, when the freezing extender contained sucrose and 1.75 M DMSO, the post-thaw sperm motility was 44% with and without egg yolk. This permits removal of the egg yolk from the freezing extender and it facilitates the evaluation of semen. Sucrose has been reported to exert a cryoprotective effect by direct interaction with the membranes, preventing freeze-thaw bilayer destabilization (Anchordoguy et al, 1988). A favourable effect of sucrose on the percentage of intact cells after freezing-thawing bull sperm has been previously observed (De Leeuw et al, 1993). A synergistic combination of sucrose and DMSO gave the most protection to sperm.

Line SY produced more sperm than lines NZ and SB. The significant differences were due to differential volume and concentration among the lines. This effect had been observed by several authors for different rabbit races and lines (Egea and Roy, 1992; Bencheikh, 1993). In addition, sperm quality, in terms of the percentage of motile sperm, normal acrosomes and sperm abnormalities, were different between the lines selected for litter size (SY and NZ) and the line selected for growth rate (SB).

The post-thaw sperm motility among the lines with individual ejaculates (52%) was comparable to that obtained by Castellini et al (1992) with a 1 M DMSO-egg yolk extender, and higher than Martin (1993) with 1.2 M DMSO-egg yolk extender. The percentage of normal acrosomes observed in this study was similar to the observation of Castellini et al (1992) for 1 M DMSO-egg yolk extender and Chen et al (1989), Castellini et al (1992) and Martin (1993) for 1 M acetamide-egg yolk extender. It was higher than Castellini et al (1992) and Martin (1993), with relatively high levels of DMSO (1.2 to 1.7 M). These results confirmed that high quality fresh semen is important to obtain good results in vitro after freezing-thawing.

When this freezing method was applied for the purpose of obtaining embryos for an embryo bank, the results were satisfactory for the line NZ. The percentage of does with normal embryos, the number of embryos by donor and the viability of embryos were not affected by the freezing semen. However, a higher number of oocytes were recovered from the frozen semen group. The 76% of the normal embryos in this group are comparable to the results obtained by Hanada and Nagase (1980) with 1 M DMSO-egg yolk extender.

In vivo viability obtained from NZ vitrified embryos are in accordance with the results of Vicente and García-Ximénez (1994) and Joly et al (1994) for vitrified and frozen embryos, respectively. These results hold promise for securing offspring from donor does.

In further studies, this freezing procedure should be evaluated with other rabbit genotypes and for its possible utilization in the programmes of conservation and on farms.

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