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Effect of freezing extender composition and male line on semen traits and reproductive performance in rabbits

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This study was conducted to elucidate the effect of different freezing extenders on two lines selected for hyperprolificacy and longevity (H and LP, respectively). In extender A, dimethyl sulphoxide (Me_2SO) and sucrose were used as cryoprotectants. In extenders B and C, the sucrose was replaced by 20% egg yolk, and in extender C the Me_2SO was substituted by acetamide. Semen was packaged in 0.25 ml plastic straws and cooled at 5°C for 45 min, and then was frozen in liquid nitrogen vapour for 10 min before being plunged into the liquid nitrogen. Thawing was carried out by immersing the straws in a water bath at 50°C for 10 s. Frozen-thawed semen characteristics and reproductive parameters were affected by freezing. Extender C showed significantly lower post-thawing quality traits than any of the three extenders. Acrosome integrity was significantly improved when Me_2SO was used as cryoprotectant. Sucrose replacement by 20% egg yolk had no effect on acrosome integrity but provided significantly lower sperm motility and viability. Freezing extender affected fertility rate, total born, number of implantation sites and gestational losses, obtaining better results when extender A was used. The acrosomal integrity after frozen-thawed process showed a significant correlation with fertility at 12^{th} day and also at birth, indicating that an increase in acrosomal integrity leads to an increase in both fertilities (12^{th} day and at birth). A positive correlation between motility of semen and implantation sites was found. The post-thawing quality traits of semen were not affected by the genetic line, although LP line showed higher total born and lower foetal and gestational losses. The findings of this study suggest that freezing extender composition has a significant effect on the success of rabbit sperm for preservation, and when Me_2SO was used as permeable cryoprotectant sucrose provided better protection compared with egg yolk and improved reproductive traits, and, on the other hand, the male genotypes used in the present study had no effect on frozen-thawed sperm parameters but negatively affected some of the reproductive parameters.

Keywords: rabbit, spermatozoa, freezing extender, genotype, fertility

Implications

Artificial insemination is widely used in rabbit breeding for meat production. The use of frozen semen would be interesting to prolong storage time and extend genetic improvement. Unfortunately, the use of frozen semen in this species is greatly limited because of its low fertility rate and/or prolificacy and variable results. There was a need to develop a freezing extender with an optimum composition to protect rabbit sperm from cryopreservation process damage and that better complies with biosafety requirements.

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Introduction

The frozen rabbit sperm has only been used for experimental or genetic resource bank purposes (Mocé and Vicente, 2009). It would be highly beneficial in routine artificial insemination (AI) programmes on commercial rabbit farms if semen could be cryopreserved without affecting fertility, although a multitude of factors affect the effectiveness of cryopreservation, including the initial quality of the semen samples, cryopreservation protocol, freezing extenders and environmental factors (Mocé and Vicente, 2009; Iaffaldano *et al.*, 2012; Lavara *et al.* 2013; Rosato and Iaffaldano, 2013). In addition, differences in reproductive parameters have been observed with fresh (Bolet *et al.*, 2000; Vicente *et al.*, 2000) and

cryopreserved sperm (Mocé *et al*., 2005) belonging to different breeds or lines of rabbits. Rabbit spermatozoa are relatively sensitive to cryoprotectants containing hydroxyl groups such as glycerol, compared with those containing amide or methyl groups (Hanada and Nagase, 1980; Chen *et al*., 1989; Castellini *et al*., 1992). In this context, the basic ingredients of current sperm-freezing extenders in rabbits are a combination of permeating cryoprotectants such as acetamide or dimethyl sulphoxide (Me_2SO) and non-permeating cryoprotective agents such as lactose, sucrose, raffinose or trehalose. In addition, egg yolk is often included as an additional protective additive in rabbit sperm-freezing extenders (Fox, 1961; Stranzinger *et al*., 1971; Mocé and Vicente, 2009).

Therefore, the aim of this experiment was to evaluate the effect of different freezing extenders on two lines selected for hyperprolificacy (H) and longevity (LP), assessing sperm parameters after thawing and reproductive performance after insemination.

Material and methods

The experiment was approved by the Ethical Committee of the Instituto Valenciano de Investigaciones Agrarias.

Animals

A total of 252 White New Zealand rabbit does (females with more than one parity without suckling any young) and 20 mature bucks belonging to two maternal lines (H and LP) were used. Line H was founded applying hyperprolific selection (Cifre *et al*., 1998), and line LP was founded applying hyperlongevity selection and an independent culling level near the average for prolificacy (Sanchez *et al*., 2008). Does and males were housed in flat deck cages with light alternating on a cycle of 16 light hours and 8 dark hours; they were fed with the same commercial diet (17.5% CP, 2.3% ether extract, 16.8% crude fibre, 2.600 kcal DE/kg) and had free access to water.

Semen collection and evaluation

Semen was collected twice a week, using an artificial vagina. A total number of 196 ejaculates was used to perform 30 heterospermic pools per line. Sperm motility, concentration, acrosome status and abnormalities were assessed as described by Lavara *et al.* (2005). Briefly, 10 μl aliquot samples from ejaculates were diluted 1 : 10 with Tris–citrate–glucose extender for a previous sperm motility rate evaluation. Ejaculates within each male line with a motility rate higher than 70% were pooled. An aliquot from each pool (10 μl) was diluted 1 : 20 in Tris–citrate–glucose extender and 10 μl of the diluted sample was placed into a 10 mm deep Makler counting chamber (Sefi Medical Instruments, Haifa, Israel) for motility analysis using a computer-assisted sperm analysis system (Sperm Class Analyzer, SCA; Microptic, Barcelona, Spain) with a specific set-up for rabbit sperm evaluation. Sperm motility was assessed at 37ºC by negative-phase contrast objective at a magnification of 100 × (NIKON E-400 microscope, Nikon Corporation Instruments Company; IZASA, Barcelona, Spain). Four microscopic fields were captured for each sample. Individual sperm tracks were visually assessed to eliminate possible debris and misdiagnosed tracks. The percentage of total motile sperm cells was recorded. A second aliquot from each pool (20 μl) was fixed with 180 ml of a 0.2% solution of glutaraldehyde in Dulbecco's phosphate-buffered saline for the acrosomal status evaluation. A minimum of 100 sperms were evaluated by phase-positive contrast microscopy at a magnification of 400 ×. Sperm concentration was determined using a Thoma–Zeiss counting cell chamber (Marienfield Laboratory Glassware, Marienfield, Germany) and expressed in terms of million of sperm per millilitre (10^6 sperm/ml).

Only semen pools with more than 70% sperm motility rate, 85% normal intact acrosomes and <15% abnormal sperm were used in this experiment.

Extenders composition and freezing–thawing procedure

All chemicals used to prepare the extenders were purchased from Sigma-Aldrich (Madrid, Spain). The solution used as a carrier for the cryoprotectants was 250 mM tris-hydroxymethylaminomethane, 83 mM citric acid, 50 mM glucose, pH 6.8 to 7.0, 300 mOsm/kg. The composition of freezing extenders is presented in Table 1. In extender A, 3 M Me_2SO and 0.1 M sucrose were used as cryoprotectants. In extenders B and C, the sucrose was replaced by 20% (v/v) egg yolk, and in extender C the Me_2SO was substituted by 2 M acetamide.

After semen evaluation, each pool was split and diluted 1 : 1 with all freezing extenders at room temperature (20ºC to 23ºC) and packaged in 0.25 ml plastic straws (IMV Technologies, L'Aigle, France). These straws were then placed at 5°C for 45 min. Cooled straws were suspended horizontally in liquid nitrogen vapour 5 cm above the liquid nitrogen surface for 10 min before being plunged in liquid nitrogen. Thawing was carried out by immersing the straws in a water bath at 50°C for 10 s.

Semen post-thawing evaluation

After the freezing–thawing process, motility and status of the acrosome were determined in the same way as for fresh semen. Hoechst stain (1 μg/100 ml; H33258, Sigma Aldrich) was used to measure viability at 25ºC to 30ºC for 15 min under fluorescence microscopy at 400 ×. Viability was determined by the percentage of sperm heads that did not stain compared with the total number of sperms examined.

AI and gestation diagnosis

A total of 252 inseminations were performed. Only receptive females (red colour of the vulvar lips) were inseminated with

Table 1 *Concentration of the cryoprotectant components of the freezing extenders*

	Freezing extender		
Cryoprotectant	A	B	C
Me_2SO	3 M	3 M	–
Acetamide	–	–	2 M
EY	–	20%	20%
Sucrose	0.1 M	–	–

Me_2SO = dimethyl sulphoxide; EY = egg yolk.

about 40×10^6 frozen–thawed sperm. Insemination was carried out using a plastic curved pipette (Imporvet, SA, Barcelona, Spain). At the same time of insemination, each female was given an intramuscular injection of buserelin acetate (1 μg; Hoechst Marion Roussel, SA, Madrid, Spain) to induce ovulation. Pregnancy diagnosis was checked by abdominal palpation 12th day after the insemination. At parturition, total born (TB) and born alive (BA) were recorded.

Embryo implantation and losses during gestation

Twelve days after AI, a total of 114 females (57 by line) were anaesthetised by intramuscular injection of 16 mg xylazine (Rompún, Bayer AG, Leverkusen, Germany), followed by an intravenous injection of 1.2 ml/kg BW of ketamine hydrochloride (Imalgène, Merial SA, Lyon, France) to maintain does under anaesthesia during ventral midline laparoscopy. The number of *corpora lutea* in both the ovaries (TO) and the number of implantation sites at 12th day in the uterine horns (IS12d) were recorded per female. Implantation and gestational losses were calculated, respectively, as the proportion of potential embryos produced (TO) that did not reach the implantation or foetal stage in all ovulating does ((TO − IS12d)/TO, (TO − TB)/TO). Foetal losses were defined as the proportion of implanted embryos that did not reach the foetal stage in pregnant does ((IS12d − TB)/IS12d). The proportions of ovulating females without implanted embryos and pregnant females that did not give birth were recorded.

Statistical analysis

A GLM including as fixed effects lines (H and LP), freezing extenders (A, B and C) and their interactions was performed with SPSS 16.0 software package (SPSS Inc., Chicago, IL, USA, 2002). For the fertility rate, a probit link with binomial error distribution was used, whereas for semen-quality traits (motility, viability and acrosome integrity), implantation sites, TB, BA and implantation, foetal and gestational losses, ANOVA was used. Pearson's correlation coefficients between frozen–thawed semen traits and fertility rate (at 12th day and at birth), implantation sites, TB and BA were calculated to determine their relationships. Values were considered statistically different at $P < 0.05$. Results were reported as least square means with s.e.m.

Results

Non-significant interactions between genetic line and freezing extender were observed for all analysed traits.

Frozen–thawed semen parameters were affected by freezing extender but not by genetic line (Table 2). The extender with acetamide and egg yolk (C) showed significantly lower post-thawing quality traits than any of the three extenders. The acrosomal integrity was significantly improved when Me_2SO was used as permeable cryoprotectant (A and B extenders). Sucrose replacement by 20% egg yolk (extender A *v.* B) had no effect in acrosomal integrity after freezing, but provided significantly lower post-thawing quality with respect to the other two.

Effects of male line and freezing extender on reproductive parameters are shown in Table 3. No significant differences were found between male genetic lines in fertility rates; only a greater number of TB were observed in line LP. However, significant differences were found in fertility rates and TB between freezing extenders (0.76 *v.* 0.52 and 0.45 fertility rate at birth and 6.5 *v.* 5.1 and 4.6 TB for extender A, B and C, respectively), obtaining better results when Me_2SO and sucrose were used as cryoprotectants (extender A).

Results obtained after laparoscopy of females are summarised in Table 4. Ovulation rate and number of implantation sites was not significantly different between male lines, but Line H showed significantly higher foetal and gestational losses. Freezing extender affected the number of implantation sites. Extender A showed the highest number and consequently the lowest implantation losses, but the gestational and foetal losses were similar among extenders.

Table 5 shows the Pearson's correlations coefficients between frozen–thawed semen characteristics and female traits. Some of the correlations between them were significant. The acrosomal integrity after frozen–thawed process showed a significant correlation with fertility at 12th day and also at birth, indicating that an increase in acrosomal integrity leads to an increase in both fertilities (12th day and at birth). In the case of sperm motility after frozen–thawed process, the obtained correlations coefficients showed that fertility at birth and the number of implanted embryos could be increased as motility increases.

Table 2 *Effect of genetic line and freezing extender on post-thaw seminal-quality parameters of rabbit sperm (LSM)*

	Line		Extender					
	H	LP	A	B	C	s.e.m.	Line *P*-value	Extender *P*-value
n	30	30	20	20	20			
Motility (%)	12.0	10.7	17.6[a]	12.2[b]	4.4[c]	0.9	0.529	<0.001
Viability (%)	14.1	12.7	18.4[a]	13.7[b]	8.0[c]	0.9	0.465	<0.001
Acrosomal integrity (%)	33.3	33.6	36.6[a]	38.5[a]	25.3[b]	1.9	0.931	0.012

LSM = least square means; H = line selected for hyperprolificacy; LP = line selected for longevity; A = freezing extender with 3 M Me_2SO and 0.1 M sucrose as cryoprotectants; B = freezing extender with 3 M Me_2SO and 20% (v/v) egg yolk as cryoprotectants; C = Freezing extender with 2 M acetamide and 20% (v/v) egg yolk as cryoprotectants; *n* = number of sperm pools used; Me_2SO = dimethyl sulphoxide.
Line × extender interaction was not significant.
[a,b]Values within a row and factor with different superscripts differ significantly at $P < 0.05$.

Table 3 *Effect of genetic line and freezing extender on reproductive parameters (LSM)*

	Line		Extender					
	H	LP	A	B	C	s.e.m.	Line *P*-value	Extender *P*-value
No. of inseminations	134	118	85	82	85			
Fertility rate at 12th day of gestation	0.62	0.72	0.80^{b}	0.64^{ab}	0.56^{a}	0.03	0.199	0.027
Fertility rate at birth	0.54	0.62	0.76^{b}	0.52^{a}	0.45^{a}	0.03	0.239	<0.001
Total born	4.8^{a}	6.1^{b}	6.5^{b}	5.1^{a}	4.6^{a}	0.3	0.019	0.008
Born alive	3.9^{a}	5.3^{b}	5.6	4.3	4.0	0.3	0.018	0.057

LSM = least square means; H = line selected for hyperprolificacy; LP = line selected for longevity; A = freezing extender with 3 M Me_2SO and 0.1 M sucrose as cryoprotectants; B = freezing extender with 3 M Me_2SO and 20% (v/v) egg yolk as cryoprotectants; C = freezing extender with 2 M acetamide and 20% (v/v) egg yolk as cryoprotectants; Me_2SO = dimethyl sulphoxide.
Line × extender interaction was not significant.
[a,b]Values within a row and factor with different superscripts differ significantly at $P < 0.05$.

Table 4 *Effect of genetic line and freezing extender on implantation rates and gestational losses (LSM)*

	Line		Extender					
	H	LP	A	B	C	s.e.m.	Line *P*-value	Extender *P*-value
n	56	57	47	34	33			
TO	10.7	10.8	10.7	10.6	10.9	0.2	0.507	0.819
IS12d	5.5	6.3	7.5^{a}	5.7^{b}	4.4^{c}	0.3	0.423	<0.001
IL	0.51	0.41	0.31^{a}	0.48^{b}	0.58^{b}	0.02	0.070	<0.001
GL	0.60^{a}	0.47^{b}	0.46	0.55	0.60	0.02	0.007	0.099
FL	0.27^{a}	0.15^{b}	0.22	0.21	0.20	0.02	0.021	0.687

LSM = least square means; H = line selected by hyperprolificacy; LP = line selected for longevity; A = freezing extender with 3 M Me_2SO and 0.1 M sucrose as cryoprotectants; B = freezing extender with 3 M Me_2SO and 20% (v/v) egg yolk as cryoprotectants; C = freezing extender with 2 M acetamide and 20% (v/v) egg yolk as cryoprotectants; *n* = number of inseminated does; TO = ovulation rate; IS12D = implantation sites at 12th day; IL = implantation losses (proportion of potential embryos produced that did not reach the implantation); GL = gestational losses (proportion of potential embryos produced that did not reach the foetal stage); FL = foetal losses (proportion of implanted embryos that did not reach the foetal stage in pregnant does); Me_2SO = dimethyl sulphoxide.
Line × extender interaction was non-significant.
[a,b,c]Values within a row and factor with different superscripts differ significantly at $P < 0.05$.

Table 5 *Pearson's correlation coefficients between frozen–thawed semen characteristics and female traits*

	Ovulation rate	Implantation sites at 12th day	Fertility rate at 12th day (%)	Fertility rate at birth (%)	Born alive	Total born
Motility (%)	0.07	0.43*	0.19	0.38*	0.26	0.32
Viability (%)	−0.07	0.11	0.18	0.20	0.02	0.10
Acrosomal integrity (%)	0.08	0.15	0.37*	0.46*	0.04	0.11

*$P < 0.05$.

Discussion

Variations in the susceptibility of semen to the cryogenic process between breeds have been reported for several species (Songsasen and Leibo, 1997; Sztein *et al*., 2000; Auerbach *et al*., 2003 in mouse; Mocé *et al*., 2003 in rabbits; Long, 2006 in poultry and Waterhouse *et al*., 2006 in boar). As our results indicate, the genetic lines used in the present study showed no differences in the cryosensitivity of semen. Although TB was significantly higher for the LP line (6.1 *v.* 4.8), and, consistently, foetal and gestational losses were significantly lower in this case. In previous studies with frozen semen in rabbit, differences in fertility rate and litter size were reported when comparing paternal and maternal lines (Mocé *et al*., 2003), but no differences were observed between maternal lines. In the present study, line LP, selected by longevity and that had prolificacy near or above the population average, seems to sustain fertility and prolificacy of frozen semen better than H line, selected by hyperprolificacy. There were no significant differences in the implantation sites in females inseminated with semen from different lines, although the lower foetal and gestational losses observed for H line resulted in a greater number of TB.

On the other hand, to protect sperm from cryopreservation process damage, the composition of the freezing extender is a key factor. Egg yolk has been shown to have protective

effects when added to an extender, but represents a risk of contamination of seminal doses and makes seminal evaluation difficult. The post-thaw sperm-quality parameters observed from different extenders used in this study show that when Me_2SO was used as permeable cryoprotectant, sucrose replacement by egg yolk had no effect in acrosome integrity after freezing, but provided significantly lower post-thawing sperm motility and viability. This finding seems to be consistent with those observed by Vicente and Viudes de Castro (1996), who reported a similar sperm motility with or without egg yolk when the extender contained sucrose. The beneficial effects of sugars in the extender on the post-thaw viability of the mammalian sperm have been reported in several studies (Abdelhakeam *et al.*, 1991; Molinia *et al.*, 1994; Vicente and Viudes de Castro, 1996; Woelders *et al.*, 1997; Malo *et al.*, 2010). Our results demonstrated that the extender with Me_2SO and sucrose, in addition to significantly improving post-thawing sperm motility and viability, enhanced the reproductive parameters, increasing the implantation sites and the fertility rate at birth, decreasing the implantation losses, and consequently, increasing the TB. However, the percentage of viability for frozen–thawed sperm observed among extenders would render the number of viable sperms in the insemination dose around 7×10^6, 5×10^6 and 2×10^6 for A, B and C extenders, respectively. These results are in agreement with those obtained by Viudes de Castro and Vicente (1997), who appreciated that does inseminated with 2×10^6 sperm showed lower pregnancy rates than females inseminated with 4×10^6 and 16×10^6 sperm. Furthermore, we found a positive correlation between acrosomal integrity and fertility and between motility of semen and implantation sites, which would explain the differences observed in fertility and litter size among them.

Regarding the use of permeable cryoprotectants, in the present study, the acetamide used in combination with egg yolk negatively affected the frozen–thawed sperm motility, contrary to the results found by Dalimata and Graham (1997). Discrepancies may be partly because of the fact that the composition of acetamide extender used by these authors (acetamide egg yolk HEPES extender with glucose, lactose and raffinose) was very different from that used in the present study (egg yolk Tris-based extender). Other small-scale experiments using 1 and 2 M Me_2SO (Hanada and Nagase, 1980 and Kashiwazaki *et al.*, 2006, respectively) showed no differences between Me_2SO and acetamide as cryoprotectants for rabbit sperm freezing. On the contrary, several authors have demonstrated the efficient sperm protection of 3.0 and 3.5 M Me_2SO in rabbit sperm cryopreservation (Vicente and Viudes de Castro, 1996; Viudes de Castro and Vicente, 1996; Mocé *et al.*, 2003; Cortell and Viudes de Castro, 2008; Iaffaldano *et al.*, 2012; Rosato and Iaffaldano, 2013). According to our results, when Me_2SO was used *v.* acetamide extender, results on frozen–thawed sperm quality and reproductive parameters studied were improved.

On the basis of our results, we may conclude that frozen–thawed sperm parameters were not affected by genetic line. However, these results support the idea that the male line may affect reproductive parameters when frozen–thawed sperm is used.

Finally, it seems that freezing extender composition has a significant effect on the cryopreservation success of rabbit spermatozoa, and when Me_2SO was used as permeable cryoprotectant sucrose provided better protection compared to egg yolk and improved reproductive traits.

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