EFFECT OF DIFFERENT FREEZING EXTENDERS ON SEMEN QUALITY, FERTILITY AND PROLIFICACY IN TWO SELECTED LINES OF RABBIT BUCKS

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ABSTRACT

The aim of this study was to evaluate the cryoprotective effect of different freezing extenders against cryopreservation injuries on rabbit sperms of 2 lines selected for hyper-prolificacy (H) and longevity (L). Ejaculates were collected and pooled from ten sexually mature rabbit bucks per each line. A total number of 196 pooled semen ejaculates per line were used to evaluate post-thawing semen by 252 artificial inseminations using White New Zealand rabbit does. Semen was equally divided into 3 volumes and diluted (1:1) with different TRIS-citric acid based extenders (A, B and C). In extender A, 3 M dimethyl sulfoxide and 0.1 M sucrose were added as cryoprotectants. For extender B, the sucrose in extender A was replaced by 20% egg-yolk, and for extender C, the TRIS-based extender was supplemented with 2 M acetamide and 20% egg-yolk. No interaction effect was detected between rabbit line and semen extender on studied traits. Moreover, no significant differences were detected between H- and L-lines for all traits except for prolificacy that was higher in L- line (6.04 *vs*. 4.37 young born at birth, respectively). However, freezing semen with extender A and B showed better post-thawing semen quality characteristics, fertility and prolificacy than frozen semen with extender C. It could be concluded that extenders A and B are preferable for freezing semen of H- and L-rabbit lines than extender C to obtain higher fertility and prolificacy. A moderate and interesting relationship was found between acrosomal integrity of frozen semen and fertility rate. (r=0.17; P=0.04).

Key words: Rabbit, semen freezing extender, semen quality, fertility, prolificacy.

INTRODUCTION

The cryopreserved semen is an excellent way to store valuable genetic materials (Bagirov, 1996; Bolet *et al.*, 2000; Blash *et al.*, 2005). However, the success of semen cryopreservation depends on several factors, including the initial quality of the semen samples, cryopreservation protocol and freezing extenders (Mocé and Vicente, 2009). They reported that Tris-based extenders (Tris, citric acid and fructose or glucose) are the base of the extenders frequently used for rabbit sperm cryopreservation. As in most species, egg yolk is commonly used in extenders for rabbit sperm freezing, its concentration varying from 10% to 20% (Fox, 1961; Stranzinger *et al.*, 1971; Mocé and Vicente, 2009).

Since 1980, amides have also been used for rabbit sperm cryopreservation (Hanada and Nagase, 1980). Amides present lower molecular weight than glycerol, and will cause less osmotic damage to the sperm. These authors reported that compounds containing hydroxyl groups seemed to be less effective cryoprotective agents for rabbit sperm than those containing amide or methyl groups.

As mentioned earlier, both fertility and prolificacy are important traits in rabbits. However, unfortunately, comparisons between extenders were always made in terms of sperm quality after cryopreservation, but few studies compared extenders using sperm fertilizing ability. Therefore, the aim of this experiment was to study the effect of different cryoprotectant agents (CPAs) in freezing extender on semen quality, fertility and prolificacy in two selected lines of rabbit bucks.

MATERIALS AND METHODS

Animals and semen collection

Semen was collected, twice a week, from 10 mature bucks (9 months of age) per line (H and L), using an artificial vagina for 10 weeks. The H- and L-lines were selected for hyper-prolificacy and longevity respectively, (Cifre *et al.*, 1998 and Sanchez *et al.*, 2008, respectively). A total number of 196 ejaculates were used to perform 10 heterospermic pools per line. Post-thawing pools were used to inseminate 252 does belonging to a maternal line selected for litter size (White New Zealand origin; Estany *et al.*, 1989). Animals were housed in individual cages, fed a commercial diet and watered ad libitum and exposure to 16L:8D photoperiod.

Freezing and thawing protocols

After collection, heterospermic pools from each line and week were equally distributed into 3 sequential tubes. Then semen per each tube was diluted 1:1 with one of 3 different isothermal glycerol free TRIS-citric acid extenders (A, B and C) at room temperature and packaged in 0.25 ml plastic straws (IMV, France). TRIS-based extender consists of 0.25 M of Tris-hydroxymethyl-aminomethane (Sigma, cat. no. T-1503), 88 mM anhydrous cirtic acid (Sigma, cat. no. C-0759), and 47 mM D-(+)-glucose (Sigma, cat. no. G-8270). In extender A, 3.0 M dimethyl sulfoxide (DMSO, Sigma, cat. no. D-5879) and 0.1 M sucrose (Sigma, cat. no. S-8501) were added as CPAs. For extender B, the TRIS-based extender was supplemented with 3.0 M dimethyl sulfoxide and 20% egg-yolk, and for extender C the basic extender was supplemented with 2 M Acetamide (Sigma, cat. no. A-0500) and 20% egg-yolk. The straws were cooled at 5°C for 45 min. To freeze sperm, straws were suspended horizontally in liquid nitrogen vapor 5 cm above the liquid nitrogen level for 10 min, before plunging into the liquid nitrogen. Semen was thawed using water bath at 45°C for 10-12 sec.

Semen post-thawing evaluation

After thawing, heterospermic pool samples were used to evaluate motility, viability and integrity as semen quality traits. Motility was examined at 37°C under a microscope with phase contrast optics, at 125x, and connected to TV monitor through a camera. Hoechst stain (1 µg/100 mL; H33258, Sigma Aldrich, Madrid, Spain) was used to measure viability at 25-30 ºC for 15 min. Viability was represented by the percentage of non-stained head sperm/total sperms. The percentage of sperm with normal apical ridge (integrity) was measured in a sample fixed with in a solution of glutaraldehyde (2%) at a magnification 400x with a differential interference contrast microscope (Nomarski contrast).

Artificial insemination

Two-hundred and fifty-two inseminations were performed. Only receptive females (red color of vulvar lips) were inseminated with about 40 million sperms of frozen-thawed semen. Insemination was carried out with a curved glass pipette (0.5 mm diameter). To induce ovulation, does were injected with 1 µg of buserelin acetate (Hoechst Marion Roussel, S.A., Madrid, Spain) intramuscularly at the time of insemination. Gestation was checked by abdominal palpation 12 days after insemination. Pregnant and non-pregnant status was noted for each doe to calculate fertility percentage. At parturition, total number of young born was recorded to calculate the prolificacy.

Statistical Analysis

The experiment was designed as a factorial randomized design (2 x 3). There were 2 rabbit lines (H vs. L) and three freezing extenders (A vs. B vs. C). Overall mean and standard error (SE) for all traits were calculated by using Means procedure (SAS, 2004). Two-ways ANOVA with interaction was performed for all traits by using the GLM procedure (SAS, 2004). Non normal distributed traits were

analyzed again using CATMOD procedure (SAS, 2004). Detected differences among main or interaction effects were tested using Tukey's honest significant differences test after ANOVA (SAS, 2004). Values were considered statistically different at $P < 0.05$.

Results were reported as least square means with standard error of the mean (SEM). The statistical model used was as follows:

$$Y_{ijk} = \mu + L_i + X_j + LX_{ij} + E_{ijk},$$

where: Y_{ijk} = the observation; k^{th} individual under i^{th} line and j^{th} freezing extender; μ = the overall mean; L_i = the effect of buck line; X_j = the effect of semen freezing extender; E_{ijk} = the uncontrolled deviation attributed to the experimental errors.

Pearson's correlation coefficients among semen quality traits (motility, viability and integrity), fertility and prolificacy were calculated by the CORR procedure of the SAS (2004). Regression coefficient equation was estimated using the REG procedure of SAS (2004).

RESULTS AND DISCUSSION

Results in Table 1 indicated no interaction effect between rabbit line and semen extender on studied traits. Moreover, no significant differences were detected between H- and L-lines except for prolificacy that was higher in L- line than H-line (6.04 *vs*. 4.37 total born at birth, respectively). However, freezing semen with extender A and B showed better post-thawing semen quality characteristics, fertility and prolificacy than frozen semen with extender C. These results are in agreement with Rohloff and Laiblin (1976) and Cortell and Viudes de Castro (2008) who compared extenders for rabbit sperm cryopreservation (Tris–citric acid–fructose, sodium citrate, Illinois Variable Temperature-IVT, MIII, etc.), none of them offering better results than the Tris-based extender. Moreover, Mocé and Vicente (2009) concluded from literature that all the tested CPAs (different amides, alcohols and DMSO), the ones that offered the best results were lactamide, acetamide or DMSO at a concentration 1 M in the extender.

Rabbit sperms present a low water permeability coefficient and a high activation energy (Curry *et al.*, 1995). This low water permeability value is consistent with the need to use CPAs of lower molecular weight and higher permeability (such as DMSO or amides) than glycerol for rabbit sperm cryopreservation (Curry *et al.*, 1995) or the egg-yolk as noted from the current trial. However, a detrimental effect of DMSO on sperm acrosomes (Hellemann *et al.*, 1979a; Martín-Bilbao, 1993) and *in vivo* fertility (Hellemann *et al.*, 1979b) has been confirmed in some studies as the concentration of this CPA increased above 4.5–5% in extenders containing egg yolk, although sperm motility increased as DMSO level increased. This negative effect of DMSO on sperm acrosomes could probably be minimized if disaccharides such as sucrose were included in the extenders (Vicente and Viudes de Castro, 1996). One M glycerol in rabbit semen extender offered the worst results, lactamide and acetamide offered the best results and DMSO gave intermediate results (Kashiwazaki *et al.*, 2006). These results were also noted when lower concentrations of CPAs were used (2% acetamide *vs*. 2% glycerol; Okuda *et al.*, 2007). Viudes de Castro *et al.* (2005) obtained results up to 64% fertility rates with 5.3 kits when rabbit semen freezing extender contained 12.4% DMSO + 0.05 M sucrose in the final mixture. Moreover, subsequent studies revealed that both sperm transport and fertility decreased when acetamide exceeded 0.83 M in the final mixture (Arriola and Foote, 2001). Results in Table 2 indicated that fertility was more correlated with sperm acrosomal integrity ($r=0.26$; $P<0.0001$).

In addition, prolificacy was more correlated with sperm motility ($r=0.17$; $P=0.04$). Regarding regression analysis the following equation was found; Fertility (%) = 0.4145 – 0.0061 Motility (%) + 0.0068 Viability (%) + 0.0072 Integrity; with $r^2 = 0.0565$ and $P = 0.0024$.

Table 1: Semen quality traits, fertility and prolificacy in response to different rabbit lines and semen freezing extenders

		Motility %	Viability %	Acrosome integrity %	Fertility %	Total born n
Overall	Mean	11.39	13.37	33.45	64	5.34
	SE[1]	1.218	1.053	1.997	3.0	0.280
Number of observations		*54*	*60*	*60*	*252*	*141*
Line effect	H-Line	12.01	14.05	33.29	60	4.37 [b]
	L-Line	10.78	12.69	33.61	68	6.04 [a]
	SEM	1.376	1.309	2.660	4.3	0.394
Freezing extender	A	18.10 [a]	18.43 [a]	36.61 [a]	79 [a]	6.30 [a]
	B	11.51 [b]	13.70 [a]	38.47 [a]	61 [ab]	4.99 [ab]
	C	4.57 [c]	8.00 [b]	25.28 [b]	52 [a]	4.32 [b]
	SEM	1.685	1.603	3.257	5.2	0.517
Interaction	H-Line*A	20.64	18.87	40.57	74	5.38
	H-Line*B	10.37	13.70	34.66	55	4.47
	H-Line*C	5.03	9.60	24.64	52	3.27
	L-Line*A	15.57	17.99	32.65	85	7.23
	L-Line*B	12.64	13.70	42.28	68	5.50
	L-Line*C	4.12	6.39	25.92	51	5.38
	SEM	2.383	2.267	4.607	7.5	0.786
Probability[2]	Line	---	---	0.9313	---	---
	Freezing extender	---	---	0.0120	---	---
	Interaction	---	---	0.2460	---	---
Chi-square[3]	Line	0.5013	0.4378	---	0.1984	0.0014
	Freezing extender	<.0001	<.0001	---	0.0002	0.0036
	Interaction	0.3904	0.6441	---	0.6181	0.7047

Means with different superscript letters on the same column within main effects differ significantly (Tukey's test; $P < 0.05$).
[1] SE = Standard error.
[2] Two-ways ANOVA with interaction using GLM procedure. Significant effect at $P < 0.05$.
[3] Chi-square using CATMOD procedure for non-normal distributed data. Significant effect at $P < 0.05$.

Table 2: Pearson's correlation coefficients among motility, viability, integrity, fertility and prolificacy

Correlation coefficient Probability *Number of observations*	Motility	Viability	Integrity
Prolificacy	0.1712	0.0981	0.08059
	0.0424	0.2472	0.3421
	141	*141*	*141*
Fertility	0.11501	0.1015	0.26329
	0.0845	0.1282	<.0001
	226	*226*	*226*
Motility		0.53081	0.48769
		<.0001	<.0001
		252	*252*
Viability			0.33007
			<.0001
			252

CONCLUSION

It could be concluded from the current study that extenders A and B are more preferable for freezing semen of H- and L-rabbit lines than extender C to obtain higher fertility and prolificacy. A moderate and interesting relationship was found between acrosomal integrity of frozen semen and fertility rate.

ACKNOWLEDGEMENTS

This work was supported, in part, by Spanish Agency for International Cooperation (AECID-PCI projects; no. A/017242/08 and A/023533/09), the project no. RTA2010-00117-00-00 from INIA and the European FEDER Funds.

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