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In vitro and in vivo fertility of ram semen cryopreserved in different extenders

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ABSTRACT

Seminal traits of frozen-thawed (FT) ram semen and in vitro and field fertility in native Portuguese breeds were evaluated in 4 experiments. In exp. 1 and 2 the cryopreservation capacity of 2 extenders, E1 (15% egg yolk-EY) and E2 (4.5% EY and trehalose) was compared through morphological evaluation and in vitro fertilizability of FT ram semen. Exp. 3 aimed to determine the usefulness of in vitro homologous/heterologous fertilization tests as tools for predicting ram fertility. Exp. 4 was conducted to verify if the identified differences between the 2 extenders could be confirmed by field fertility. E1 showed a better cryoprotective action expressed by higher in vitro and field fertility results. In conclusion, EY is difficult to be replaced in ram semen extenders. Heterologous fertilization seems to be a useful tool for predicting fertility of FT ram semen.

1. Introduction

Low pregnancy rates after cervical insemination (AI) with frozen spermatozoa (spz) are responsible for the scarcely widespread of ram AI. A defective antioxidant capacity associated with high unsaturated:saturated fatty acids ratio and low cholesterol content of sperm plasma membrane have a major influence in the extensive damages inflicted to ram spz during cryopreservation. Tris-EY based diluents have been reported to provide adequate cryoprotection (Salamon and Maxwell, 2000). However, due to EY variability it is quite difficult to reproduce trials and transmissible infectious agents can be introduced by this cryoprotectors. Partial or total EY substitution in ram extenders has been tried (Partida et al., 1998; Matsuoka et al., 2006). Trehalose, because of its cryoprotectant and antioxidant properties, was included in semen extenders enhancing post-thaw fertility (Aisen et al., 2002).

On the other hand, homologous and heterologous in vitro fertilization (IVF) have already been used to assess sperm fertility with good results (Choudhry et al., 1995; Morris et al., 2001). Heterologous IVF is an attractive evaluation method because it does not require the use of homologous gametes, which are difficult to find in our local abattoirs and is less expensive and labour intensive than field fertilization assays.

Our objective was to compare an extender containing trehalose and a lower EY concentration with the extender normally used in our lab, through the evaluation of seminal traits of FT ram semen, in vitro and field fertility in native Portuguese breeds. The usefulness of homologous/heterologous IVF tests as tools for predicting FT ram semen fertility was also determined.

2. Materials and methods

2.1. Experimental design

This work consisted of 4 experiments. In exp. 1, the objective was to compare 2 distinct extenders (E1 *vs.* E2) through morphological and thermoresistance evaluation

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Table 1
Morphological parameters evaluation of fresh and thawed ram semen diluted in two extenders (extender 1 – E1 and extender 2 – E2).

Characteristics	Semen status	Extender	
		E1	E2
Motility (%)	Fresh	67.8 ± 2.3[a]	68.8 ± 2.3[a]
	Thawed	46.5 ± 5.3[a]	36.9 ± 11.1[b]
Viability (%)	Fresh	84.5 ± 7.1[a]	85.9 ± 5.9[a]
	Thawed	59.8 ± 10.8[a]	66.5 ± 10.3[b]
Normality (%)	Fresh	85.4 ± 5.2[a]	86.9 ± 5.4[a]
	Thawed	78.3 ± 5.9[a]	80.3 ± 5.4[a]
Acrosome integrity (%)	Fresh	88.8 ± 5.3[a]	89.7 ± 4.7[a]
	Thawed	80.1 ± 8.5[a]	79.0 ± 9.1[a]
HOS test (%)	Fresh	80.4 ± 11.9[a]	85.1 ± 8.2[a]
	Thawed	40.6 ± 15.2[a]	32.1 ± 11.8[b]

Mean ± S.D. Data within the same lines with different superscripts letters are statistically different ($P \leq 0.05$).

of FT semen (2 rams, 14 ejaculates each, 168 straws). In vitro fertilizing capacity of spz frozen with E1 and E2 was determined in exp. 2. Ejaculates from the same collection (2 rams, 3 ejaculates per ram, 24 straws) were used for capacitation, heterologous and homologous IVF and embryo production assessment (6 sessions). Exp. 3 assessed the usefulness of homologous/heterologous IVF tests as tools to predict FT ram semen fertility. Ejaculates from the same collection (2 rams, 3 ejaculates per ram, 48 straws) were used for morphological and post-swim-up FT semen evaluation. An equation for predicting the effect of studied variables on embryo production was tested. Exp. 4 was designed to determine if differences between the 2 extenders (E1 *vs.* E2), identified via IVF evaluation, could be confirmed by field fertility achieved after cervical AI (2 rams, 151 ewes, 302 straws).

2.2. Semen collection, cryopreservation and morphological evaluation

Ejaculates, collected from Portuguese Serra da Estrela ($n = 2$) and Saloia ($n = 2$) rams by artificial vagina, were immediately evaluated and semen with poor quality rejected. Two extenders were used to dilute each ejaculate: E1, currently used in our lab (15% EY, Marques et al., 2006), in a single fraction and E2 composed by 2 fractions in equal volumes, fraction A (25.6 g L^{-1} Tris, 13.2 g L^{-1} citric acid, 9.4 g L^{-1} fructose, 9.4 g L^{-1} glycine and 9.4% EY) and fraction B (fraction A without EY plus 5.3% glycerol and 66.8 g L^{-1} trehalose). Semen was packed (1.2×10^9 spz mL^{-1}) in mini-straws and frozen in NL_2. Fresh and thawed semen samples were evaluated for individual motility (IM), viability and abnormalities (nigrosin-eosin). Acrosome integrity and hypoosmotic swelling test (HOST) were assessed as in Cortes et al. (2006). For thermoresistance evaluation, E1 and E2 thawed semen were diluted in physiologic serum and maintained in a thermostatic bath at 37.5 °C. The IM was evaluated immediately after thawing and every 10 min during 1 h (Aisen et al., 2002).

2.3. Post-swim-up semen evaluation

After IM evaluation, FT semen was incubated at 38.5 °C and 5% CO_2 during 1 h. Post-swim-up sperm IM, concentration and capacitation status (chlortetracycline staining-CTC) were determined. For homologous IVF, oocytes collected from slaughterhouse ovine ovaries were matured (22 h) and co-cultured with swimed-up spz (1×10^6 mL^{-1}) for 18 h (Pereira et al., in press). Samples of presumptive zygotes were stained (1% aceto-lacmoid). Fertilization was considered to occur by the observation of a decondensed sperm head, 2 pro-nuclei or zygotes and polyspermy by observing more than 2 swollen sperm heads or 2 pronuclei within a single oocyte. The remaining zygotes were cultured at 38.5 °C, 5% O_2, 5% CO_2 and 90% N_2 until ecloded blastocyst stage. For heterologous IVF, bovine oocytes were matured for 22 h (Cortes et al., 2006) and inseminated with ovine spz. Presumptive zygotes (20 h) were fixed and evaluated as in homologous IVF.

2.4. Artificial insemination/field fertility

Multiparous Saloia ewes ($n = 151$, 5–6 years old, 3–4 parturitions) were synchronized in May with intravaginal 40 mg fluorogestone acetate sponges (Chronogest®, Intervet) during 12 days and 500 IU eCG (i.m., Intergonan®, Intervet) at sponge withdrawal. Cervical insemination with 0.6×10^9 FT spz per ewe was performed 54–55 h after sponge removal. Fertility rate was determined as the total number of ewes lambing supervised by the farmer over the total number of ewes inseminated.

2.5. Statistical analysis

Statistical differences involving multiple treatments were determined by ANOVA/MANOVA including extender

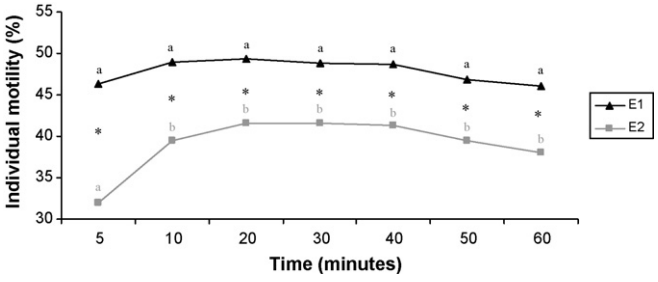

Fig. 1. Post-thawing thermoresistance (37.5 °C) of ram semen diluted with extender 1 (E1) or extender 2 (E2). Different letters within extenders (a and b) are statistically different ($P \leq 0.05$). Between extenders all means (*) are statistically different ($P \leq 0.05$).