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 in 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.

Keywords: IVF, cervical insemination, ram, frozen semen, homologous and heterologous fertilization

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 in 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 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 gL⁻¹ Tris, 13.2 gL⁻¹ citric acid, 9.4 gL⁻¹ fructose, 9.4 gL⁻¹ glycine and 9.4% EY) and fraction B (fraction A without EY plus 5.3% glycerol and 66.8 gL⁻¹ trehalose). Semen was packed (1.2x10⁹ spz mL⁻¹) in mini-straws and frozen in NL₂. 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 1h (Aisen et al., 2002).

2.3. Post-swim-up semen evaluation

After IM evaluation, FT semen was incubated at 38.5 °C and 5% CO₂ during 1h. 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 (22h) and co-cultured with swimed-up spz (1×10⁶ mL⁻¹) for
18h (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 prounuclei within a single oocyte. The remaining zygotes were cultured
at 38.5 °C, 5% O₂, 5% CO₂ and 90% N₂ until ecloded blastocyst stage. For heterologous
IVF, bovine oocytes were matured for 22h (Cortes et al., 2006) and inseminated with
ovine spz. Presumptive zygotes (20h) 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 0.6×10⁹ FT spz per ewe was performed 54-55h 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 composition and rams as main effects. Data from homologous/heterologous IVF status were analysed by 2x2 Chi Square using Fisher’s exact test. Pearson’s coefficients of correlation were calculated to determine the relationship among variables. Stepwise multiple regression analysis were used to establish a regression equation able to predict fertility (embryo production rate) on the basis of semen or IVF traits/parameters. Only variables presenting a significant Pearson’s correlation coefficient with embryo production were used. A value of $P \leq 0.05$ was chosen as an indicator of significance (Statsoft Inc., 1995).

3. Results

In all experiments no differences ($P > 0.05$) were found between rams or significant interactions ($P > 0.05$) between rams and extender composition in the evaluated parameters. Ram effect was eliminated from the model.

3.1. Experiment 1

Thawed E1 semen presented an increased motility ($P = 0.001$) and better HOST ($P = 0.03$) than E2 semen (table 1), but spz viability was better ($P = 0.02$) in E2 semen. Thermoresistance was always higher in E1 FT semen (figure 1, mean IM $P \leq 0.001$).
3.2. Experiment 2

Pre (P=0.004) and post (P=0.003) swim-up IM were higher in thawed E1 semen (table 2). No differences were identified in post-swim-up spz concentration and capacitation. Homologous IVF rate was higher in E1 semen (P=0.04, figure 2), presenting an advanced stage of fertilization with more 2 pronuclei and less decondensed sperm heads. E1 semen increased polyspermy (P=0.003), cleavage rates (P=0.02, table 2) and also heterologous IVF rate (P=0.05, figure 2) advancing fertilization stages (P=0.003).

3.3. Experiment 3

Exception made for IM, none of seminal parameters nor CTC results were correlated with embryo production rates. Among correlations found to be significant (table 3), it was possible to established a linear regression equation \( Y = -11.51 + 0.45 X; r^2=0.3522 \) and \( P=0.04 \) between heterologous IVF (X) and D7/8 embryo rates (Y), thus predicting D7/8 embryo rate based on known heterologous IVF data.

3.4. Experiment 4

Field fertility of ewes after AI was higher with E1 FT semen (20.5 vs. 3.8 %, \( P<0.001 \)).

4. Discussion

Partial or even total replacement of EY in ram extenders has already been tried with
different levels of success (Partida et al., 1998, Matsuoka et al., 2006). In our study, although E2, providing lower EY levels (4.5%), did not affect morphological normality of FT semen, higher thermoresistance, pre and post swim-up IM and homologous/heterologous IVF rates were obtained with E1 (15% EY). These superior results were confirmed by field fertility. Moreover, in E2, trehalose (Aisen et al., 2002) and glycine supplementation allied to replacement of glucose by fructose, which is more easily metabolized by sperm, were not able to compensate the experimental EY insufficiency. According to our results the extender composition had a major effect on post-thawed semen viability. Although using only four rams, repeated measures from each subject were performed. By splitting ejaculate from each ram in two fractions prior to dilution with E1 and E2, each ram or (ejaculate from each ram) was its own control. Unlike other authors that referred a significant male effect on semen freezability (Marques et al., 2006), in each experiment no differences (P>0.05) were found between rams or interactions (P>0.05) between rams and extender composition for the evaluated parameters.

Selection of the most adequate fertility prediction parameter has been the goal of late research. HOST (Matsuoka et al., 2006) and sperm motility analyses (Morris et al., 2001) were recognized as useful methods. Although some authors were unable to relate in vivo fertility with in vitro sperm functional tests or homologous IVF (Papadopoulos et al., 2005), in the present study, a relatively high correlation between post-thaw semen IM and homologous IVF or cleavage rates was obtained. However, homologous IVF procedures as used routinely are designed to maximize blastocysts yields rather than detecting differences in sperm fertilizing ability, although lowering in vitro spz doses could be a solution (O’Meara et al., 2005). Heterologous IVF tests could be an alternative to identify differences between individual rams (Choudhry et al., 1995). In
our study, it was possible to establish a regression equation ($Y= -11.51 + 0.45 X$; $r^2=0.3522$ and $P=0.04$) able to predict embryo production rate ($Y$) based on known heterologous IVF data ($X$). Nevertheless, further work on a larger scale is needed to make these predictions more accurate. In conclusion, present results demonstrate that the EY presence is difficult to be replaced in ram semen extenders. Heterologous IVF may be used as a tool to predict the potential fertility of FT semen.

References


the characteristics of frozen-thawed ram spermatozoa and in vitro embryo production.


Figure 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 ≤ 0.05). Between extenders all means (*) are statistically different (P ≤ 0.05).
Figure 2: Assessment of homologous and heterologous in vitro fertilization (IVF) with semen frozen in two different extenders (E1 and E2).

Unfert: unfertilized oocyte; Decond: sperm head decondensation; 2 pro: 2 pronuclei; Polys: Polyspermy;

Fert rate: fertilization rate; * P ≤ 0.05 within the same IVF type
Table 1. Morphological parameters evaluation of fresh and thawed ram semen diluted in two extenders (extender 1 - E1 and extender 2 - E2).

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Semen status</th>
<th>Extender E1</th>
<th>Extender E2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Motility (%)</td>
<td>Fresh</td>
<td>67.8 ± 2.3a</td>
<td>68.8 ± 2.3a</td>
</tr>
<tr>
<td></td>
<td>Thawed</td>
<td>46.5 ± 5.3a</td>
<td>36.9 ± 11.1b</td>
</tr>
<tr>
<td>Viability (%)</td>
<td>Fresh</td>
<td>84.5 ± 7.1a</td>
<td>85.9 ± 5.9a</td>
</tr>
<tr>
<td></td>
<td>Thawed</td>
<td>59.8 ± 10.8a</td>
<td>66.5 ± 10.3b</td>
</tr>
<tr>
<td>Normality (%)</td>
<td>Fresh</td>
<td>85.4 ± 5.2a</td>
<td>86.9 ± 5.4a</td>
</tr>
<tr>
<td></td>
<td>Thawed</td>
<td>78.3 ± 5.9a</td>
<td>80.3 ± 5.4a</td>
</tr>
<tr>
<td>Acrosome integrity (%)</td>
<td>Fresh</td>
<td>88.8 ± 5.3a</td>
<td>89.7 ± 4.7a</td>
</tr>
<tr>
<td></td>
<td>Thawed</td>
<td>80.14 ± 8.5a</td>
<td>79.0 ± 9.1a</td>
</tr>
<tr>
<td>HOS test (%)</td>
<td>Fresh</td>
<td>80.4 ± 11.9a</td>
<td>85.1 ± 8.2a</td>
</tr>
<tr>
<td></td>
<td>Thawed</td>
<td>40.6 ± 15.2a</td>
<td>32.1 ± 11.8b</td>
</tr>
</tbody>
</table>

Means ± S.D. Data within the same lines with different superscripts letters are statistically different (P ≤ 0.05).
Table 2. Post-thawed sperm quality parameters and embryo production yields evaluation according to semen extender (extender 1 - E1 and extender 2 - E2).

<table>
<thead>
<tr>
<th>Extender</th>
<th>Thawed Motility (%)</th>
<th>Post swim-up Motility (%)</th>
<th>Inseminated oocytes (n)</th>
<th>Cleavage rate (%)</th>
<th>D7/D8 Embryo rate (%)</th>
<th>Ecloded embryo rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E1</td>
<td>57.1 ± 13.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>67.9 ± 5.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>250</td>
<td>57.2 ± 7.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>19.2 ± 13.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>27.8 ± 27.7&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>E2</td>
<td>42.1 ± 8.9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>56.7 ± 10.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>273</td>
<td>33.5 ± 20.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>8.8 ± 5.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8.3 ± 20.4&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Means ± S.D., Motility – individual spermatozoa motility; Embryo productions expressed as percentages of inseminated oocytes or cleaved embryos, for cleavage and D7/D8 or ecloded embryo rates, respectively.

Data within the same columns with different superscripts letters are statistically different.
Table 3. Pearson’s ranks correlations (r) between parameters of embryo production.

<table>
<thead>
<tr>
<th>Outcome variable</th>
<th>Parameter of embryo production</th>
<th>r</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cleavage rate</td>
<td>pre swim-up mobility</td>
<td>0.76</td>
<td>0.0001</td>
</tr>
<tr>
<td></td>
<td>post swim-up mobility</td>
<td>0.76</td>
<td>0.004</td>
</tr>
<tr>
<td></td>
<td>Homologous fertilization rate</td>
<td>0.89</td>
<td>0.0001</td>
</tr>
<tr>
<td></td>
<td>Homologous 2 pronucleos</td>
<td>0.7</td>
<td>0.012</td>
</tr>
<tr>
<td></td>
<td>Homologous polyspermy</td>
<td>0.75</td>
<td>0.005</td>
</tr>
<tr>
<td></td>
<td>Homologous unfertilized oocytes</td>
<td>-0.89</td>
<td>0.0001</td>
</tr>
<tr>
<td>Day 7/8 embryo rate</td>
<td>Heterologous unfertilized oocytes</td>
<td>-0.59</td>
<td>0.04</td>
</tr>
<tr>
<td></td>
<td>Heterologous fertilization rate</td>
<td>0.60</td>
<td>0.0001</td>
</tr>
</tbody>
</table>