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Effect of pasteurized egg yolk and rosemary honey supplementation on quality of cryopreserved ram semen

R.A. Jerez-Ebensperger[a], V. Luño[a], M. Olaciregui[a], N. González[a], I. de Blas[b], L. Gil[a,*]

[a] Reproduction and Obstetrics Area, Animal Pathology Department, Universidad de Zaragoza, Spain
[b] Infectious Diseases Area, Animal Pathology Department, Universidad de Zaragoza, Spain

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ABSTRACT

The aim of this study was to determine the effect of pasteurized egg yolk and the supplementation with rosemary honey on ram sperm quality after cryopreservation process. Ejaculates were obtained from three rams (Rasa Aragonesa), and semen samples were processed using straw freezing procedure. In experiment 1, semen samples were frozen in four different extenders: (D1) Fiser egg yolk extender as control, and three experimental extenders: (D2) pasteurized egg yolk in fraction 1 (F1); (D3) rosemary honey in F1 and fraction 2 (F2); D4) pasteurized egg yolk and rosemary honey in F1 and honey in F2. Total and progressive motility, viability, acrosome integrity and hypoosmotic swelling test response (HOST test) were evaluated post-thawing and 2 h post-thawing. A significant increase in percentage of total motile sperm was observed in the presence of pasteurized egg yolk, alone or in combination with rosemary honey. Acrosome integrity was significantly higher in pasteurized egg yolk extenders ($p < 0.05$), however rosemary honey decreased HOST positive sperm after thawing. In experiment 2, embryo development was tested. D2 extender showed that the embryo development rate was significantly ($p < 0.05$) higher than both D1 and D4 extenders. It could be suggested that Fiser extender composition can be modified replacing fresh egg yolk by pasteurized egg yolk and fructose by honey, without detrimental effects on sperm quality after cryopreservation.

1. Introduction

Cryopreservation as a technique for storing ram semen has many advantages but during freeze-thawing process thermal, mechanical, chemical and osmotic stress (Holt et al., 1992; Holt and North, 1994; Watson, 1981) induce certain detrimental structural effects on spermatozoa. These changes involve several biochemical and functional damages (Bailey et al., 2000; Watson, 2000) to the spermatozoa that reduce their motility, plasma membrane functionality and acrosome integrity (Celeghini et al., 2007; Woelders et al., 1997), leading spermatozoa to a lower fertilizing ability (Medeiros et al., 2002; Salamon and Maxwell, 2000; Tekin et al., 2006).

Maintenance semen quality during cryopreservation is the main objective of the process. The design and use of suitable extenders and protocols is an important point to avoid the adverse effects of cold shock, such as intracellular ice crystals formation. The utilization of egg yolk in the semen extender was reported in 1940, Philips and Lardy (1940) indicated that egg yolk was beneficial for sperm preservation. Currently egg yolk is a usual component in most of the sperm cryopreservation extenders in domestic and exotic animals. Egg yolk has a beneficial effect as stabilizer of the spermatozoa membrane (Salamon and Maxwell, 2000) and acrosome integrity in association with other components (Bogart and Mayer, 1950; Phillips, 1939), and protects against cold shock (Drobnis et al., 1993; Watson, 1981). Furthermore, the addition of sugars to extenders shows a beneficial effect on spermatozoa viability and acrosome integrity (Martín et al., 1980). Rosemary honey provides a combination of large amount of sugars (including glucose, fructose and sucrose) with antioxidant properties (Alamanni, 1994). The combination of both characteristics may protect at spermatozoa against cold shock injuries.

In order to determine new alternatives in cryopreservation protocols, we investigated the role of pasteurized egg yolk and rosemary honey on ram sperm quality during cryopreservation.

* Corresponding author at: C/Miguel Servet 177, 50013 Zaragoza, Spain. Fax: +34 976 761612.
E-mail address: lydiagil@unizar.es (L. Gil).

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2. Materials and methods

2.1. Animals and semen collection

Semen was collected from three Rasa Aragonesa rams, aged 3–4 years, in the breeding season (October–December) with a twice-week seminal collection rate using the artificial vagina technique.

2.2. Semen dilution, cryopreservation and thawing

All chemicals were obtained from Sigma Aldrich, unless otherwise indicated. Fresh semen samples were examined for mass movement, motility, vigor and concentration. Only ejaculates with movement greater than 3 (Evans and Maxwell, 1987) and sperm concentration more than 2.5×10^9 sperm/ml were accepted. In each replica, three ejaculates were pooled and diluted to final concentration of 100×10^6 sperm/ml.

The medium described by Fiser et al. (1987) was used as the base and control extender (D1). This extender consisted of two parts: F1 (269 mM Tris, 89 mM citric acid), 52 mM D-fructose, 25% egg yolk (v/v), 2% glycerol (v/v) and F2 (26.66 mM sodium citrate, 6.89 mM TES, 3.62 mM glycine, 295.30 mM lactose, 23.51 Mm raffinose, 28.09 mM fructose, 34 mM dextran; 12% of the obtained solution was replaced by the same volume of glycerol). In order to test pasteurized egg yolk and rosemary honey, these were added to the F1 and F2 fraction of the Fiser extender. The three experimental extenders were: D2, 25% (v/v) pasteurized egg in F1 instead egg yolk; D3, 5% (v/v) honey in F1 and F2 instead D- fructose; D4, 25% (v/v) pasteurized egg and 5% (v/v) honey in F1 instead egg yolk and D- fructose and 5% (v/v) honey in F2 instead D- fructosa. Osmolarity was adjusted to 338mOsm/ml and pH to 7.5.

The amount of sugars contained in the rosemary honey was determined by HPLC (high-performance liquid chromatography) technique, with following results: 24.5 ± 0.1 g/l of fructose and 23.9 ± 0.1 g/l of glucose.

Freezing procedure was based on the method described by Fiser et al. (1987). After collection, semen was diluted with fraction 1 from each extender. Temperature of sperm samples decreased gradually from 30 °C to 5 °C during 1 h. Then, fraction 2 was added and samples were maintained at 5 °C for 2 h for stabilization before freezing. Samples were packed in 0.25 ml plastic straws, placed into a freezer unit, at 4 cm from the level of liquid nitrogen during 10 min. The straws were then plunged into liquid nitrogen (−196 °C) and stored until thawing. After 1 day, semen straws were thawed in a water bath (37 °C, 21 s) and sperm were evaluated.

2.3. Semen quality evaluation

Total and progressive motility sperm were assessed using ISAS® system (PROISER). Sperm viability was determined by nigrosin-eosin staining (Eliasson and Treichl, 1971). Acrosome integrity was evaluated with the method described by Pursel and Johnson (1974). Membrane integrity (Host test) was assessed after incubation of sperm sample in sodium citrate (100mOsm/kg) at 37 °C for 30 min, and then the samples were fixed in glutaraldehyde for further evaluation. Semen samples were evaluated after thawing at 0 and 2 h incubation at 37 °C.

2.4. In vitro oocytes maturation, fertilization and embryo culture

Sheep ovaries were obtained from a local slaughterhouse and transported in SSF containing 250 IU/mL penicillin at 30 °C. At the laboratory, the ovaries were washed three times in SSF. Oocytes were aspirated from follicles with 2–6 mm diameter using a 19-gauge needle attached to a 2 ml syringe containing 1 ml of aspiration medium Phosphate Buffered Saline (PBS) supplemented with heparin (0.45 mg/ml) and penicillin-streptomycin (0.05 mg/ml). Only oocytes with complete cumulus (COCs) layers and homogeneous cytoplasm were selected. After classification, oocytes were washed three times in the maturation medium: TCM199 supplemented with sheep serum (10% v/v), FSH/LH (7.5 UI/ml), glutamine (270 μg/ml), sodium pyruvate (41 μg/ml), cysteamine (7.7 μg/ml) and penicillin- streptomycin (0.05 mg/ml). Oocytes were transferred in groups (20–30/drop) into 50 μl droplets of maturation medium and incubated for 24 h at 38.5 °C in a humidified 5% CO_2 atmosphere under mineral oil.

Previously fertilization, oocytes were denuded in a vortex during 2 min. Oocytes were washed three times in fertilization medium and transferred in groups of 20–30 into 50 μL droplets of fertilization medium: SOF supplemented with sheep serum (10% v/v), calcium lactate (32 μg/ml) and gentamicin (20 μg/ml).

Before fertilization, highly motile spermatozoa were selected by swim-up technique. Frozen sperm samples from D1, D2 and D4 groups, were thawed at 37 °C 21s and diluted in capacitation medium (SOF supplemented with 12.38 mg/ml HEPES, 4 mg/ml BSA and 25 μg/ml gentamicin). Sperm samples were centrifuged at 1500 g × 5 min. Supernatant were remove, and the same volume that sediment was completed with capacitation medium. This suspension was incubated during 45 min in a humidified 5% CO_2 atmosphere at 38.5 °C. Sperm suspension was adjusted at final concentration of 1×10^6 sperm/ml and added into each fertilization droplet. The sperm and oocytes were incubated for 24 h at 38.5 °C in a humidified 5% CO_2 atmosphere.

Presumptive zygotes were removed from fertilization medium, washed three times in SOF medium and transferred to a culture medium (SOF added with 20 μl/ml BME, 10 μl/ml MEM, 55.5 μl/ ml SFB, 54.75 μg/ml glutamine, 8 μg/ml BSA and 40 μg/ml gentamicin) during 48 h under mineral oil, at 38.5 °C in 5% CO2.

2.5. Assessment of embryo development

To evaluate in vitro embryo development rate, Hoechst 33342 stain was used. Embryos were fixed with 1% glutaraldehyde in PBS for 30 min at room temperature and stained with Hoechst 33342. In order to evaluate embryo development, the rate of 8 cells formation at 72 h after insemination was determined.

2.6. Statistical analysis

Statistical analyses were performed using the SPSS package, version 17.0 for Windows (SPSS Inc., Chicago, IL, USA). A Kolmogorov-Smirnov test was realized to determine if the data were normally distributed. Total and progressive motility, viability, Host test, acrosomal status data were analysed by analysis of variance (ANOVA). When ANOVA revealed a significant effect, values were compared by the least significant difference pairwise multiple comparison post hoc test (Duncan). Results were expressed as mean ± standard error of the mean. The penetration and cleavage rates were analyzed using the Chi-square test and data were expressed in percentages. Differences were considered statistically significant at $p < 0.05$.

3. Results

3.1. Experiment 1: effect of experimental freezing extenders on sperm quality

The effects of different extenders on semen quality at different time are reported in Table 1. There were significant differences among extenders in total motility at 0 h and 2 h post-thawing. A significant increase in percentage of total motile sperm was observed in the presence of pasteurized egg yolk, alone or in combination