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Use of soy milk combined with different cryoprotectants for the ram semen cryopreservation

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ABSTRACT

The aim of this study was to determine the effect of based milk soy extender combined with two different cryoprotectants: dimethylformamide (DMF) and glycerol, on ram sperm quality after cryopreservation. Five freezing extenders were assessed: Tryladil, Andromed (controls) and three soya milk experimental extenders containing: (A) 6% glycerol, (B) 3% DMF and 3% glycerol and (C) 6% DMF. Semen samples were assessed at post dilution, prefreezing, post thawed and at 2 h after thawing. *In vitro* fertilization was performed with two selected extenders based on thawed semen quality. After thawing, semen samples showed a significant ($P < 0.001$) higher percentages of motility in presence of DMF: (B) 36.60%, (C) 41.00% *vs* Tryladil 28.12%, Andromed 26.32% and (A) 29.60%. However, 2 h post thawing the extenders containing DMF produced a significant ($P < 0.001$) negative effects on motility and acrosome integrity rates. Embryo production rates were significantly higher ($P = 0.006$) when fertilization was performed with semen frozen with Andromed (75.5% 8–16 cell embryos) compared to (A) (56.2% 8–16 cell embryos). In conclusion, the combination of soy milk and glycerol is suitable for ram semen cryopreservation, better than that with DMF.

1. Introduction

Cryopreservation process produces significant changes in mammalian spermatozoa with the subsequent decrease in its fertility potential. Different factors are involved in the alteration of sperm during the cryopreservation: the maturity of the cells, the cryoprotectant used and chilling, freezing and thawed processes employed. Therefore, a large number of extenders have been studied in order to preserve the semen quality during the cryopreservation (Hammadeh et al., 1999). Skimmed milk is the most widely used extender for preserving ram semen (Jonhson et al., 1974), although in the last decade many studies were performed on plant-based extenders, like soya milk for chilling (Meque, 2004) and freezing (Mualuzanga, 2005) ram semen. In addition, cryoprotectants are incorporated to the extender to avoid the adverse effects of cold shock and prolong the viability and functionality of sperm cells. Glycerol has been the first cryoprotectant used (Polge et al., 1949) showing a positive effect on the cells survival during freezing processes, and it has been routinely used to compound the freezing extender for semen of many species. However, it could causes injury to spermatozoa during cryopreservation process (Fahy et al., 1990). Glycerol produces toxicity on sperm cell sample, partly due to the osmotic stress because it permeates the cell membrane slower than other cryoprotectants, and increases intracellular viscosity (Gilmore et al., 1995). Furthermore, this cryoprotectant may cause the denaturation of the proteins in cell cytoplasm (Demick et al., 1976; Pace and Sullivan 1975). Therefore its use as a cryoprotectant could be a factor involved in poor post-thaw motility and fertility rates in frozen stallion doses (Alvarenga et al., 2003). Negative effects of glycerol have encouraged investigation on alternative ways to reduce its toxicity effect. The optimum concentration of glycerol in extenders has not still been determined, although comparative studies have been performed in different species: ovine (Bailey et al., 2000), canine (Futino et al., 2010) and porcine (Buranaamnuay et al., 2011). Different studies have been performed on stallion semen to determine the cryoprotective effect of different substances with similar properties but with less toxic effects (Alvarenga et al., 2005; Medeiros et al., 2002). In fact, the dimethylformamide (DMF) molecular weight is obviously lower than glycerol, what has been suggested is that this property might induce less osmotic damage. Studies performed on boar semen

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showed that the combination of DMF and dimethylacetamide at 5% concentration increased the sperm quality after thawing (Bianchi et al., 2008).

Therefore, the objective of this study was to evaluate whether DMF might replace partly or totally the glycerol in soya milk ram semen extenders based on ram semen quality. In addition we performed the in-vitro fertility test of those treatments from first trial considered valuable for further assessment in a second trial.

2. Material and methods

2.1. Reagents and media

Unless otherwise indicated, all chemicals were obtained from Sigma–Aldrich Co. (Alcohobendas, Madrid, Spain). Andromed and Tryladil were from Minitübe (Germany). Soya milk was from Nutrifoods-Consult. e Com de Prod. Alimentares, Lda (Portugal). DFM was from Panreac (S.L.U, Barcelona, Spain) and pluset was from Laboratorios Calier (Barcelona, Spain).

2.2. Extenders

In the present study for freezing ram semen, five extenders were used. Two commercial extenders as controls: Andromed and Tryladil, and three experimental extenders. Andromed contains phospholipids, TRIS, citric acid, sugars, antioxidants, buffers, glycerin, and antibiotics. Tryladil contains TRIS, citric acid, sugar, buffers, glycerin, tylosin, gentamicin, spectinomycin and lincomycin. Both of them reconstituted with ultrapure water.The experimental extenders were performed with a common base (0.1 g/ml soya skim milk, 0.01 g/ml glucose, 0.001 g/ml streptomycine, 0.02 g/ml lactose) and varying the concentration and type of cryoprotectant: (A) 6% glycerol, (B) with 3% glycerol and 3% DMF, (C) with 6% DMF. Osmolarity ans pH was assessed before addition of cryoprotectants, and was adjusted to 300 mOsmol and 7.3 respectively.

2.3. Animals, semen collection and cryopreservation

Semen was collected from three Rasa Aragonesa rams, aged 3–4 years, in the breeding season (October–December) in a twice a week seminal collection regime using an artificial vagina. 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 ejaculates from three rams were pooled and divided into five aliquots including controls and three test groups. Concentration was evaluated using the concentration module of a computer assisted semen analysis (CASA) (ISAS® Proiser, Valencia, Spain), and then the corresponding extender was added in the volume necessary to reach a final semen concentration of 100×10^6. Then semen samples were cooled to 4 °C in 2 h, 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.

2.4. Semen evaluation

Total and progressive motility sperm were assessed using the CASA system, analyzing five fields by sample (200 sperms/field). Sperm viability was determined by eosin–nigrosin staining. A semen sample was diluted 1:1 (v/v) with stain solution (5% eosin, 10% nigrosin in a citrate solution) and smeared. Live spermatozoa remained unstained. Acrosome integrity was evaluated by the method described by Pursel and Johnson (1974). Membrane integrity (Host test) was assessed after incubating 10 μl of semen with 90 μl of citrate hypoosmotic solution (100 mOsm/kg) at 37 °C for 30 min, and then samples were fixed in 8% glutaraldehyde for further evaluation. The proportion of sperm with swollen or coiled tails was considered as HOST-positive. These parameters were evaluated immediately after dilution, pre freezing (after equilibration), post thawing and 2 h after incubation at 37 °C (thermoresistance test).

2.5. Oocytes collection and in vitro maturation

Oocytes were obtained from ovaries of adult sheeps at a local slaughterhouse and transported to the laboratory at 35 °C in Phosphate Buffered Saline (PBS, D 4031) within 1 h. At the laboratory, the ovaries were washed three times in physiological saline (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 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 (Sigma M7528) supplemented with sheep serum (SS 10% v/v S2263), 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 μ l 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 to 30 into 50 μL droplets of fertilization medium: SOF supplemented with SS (10% v/v), calcium lactate (32 μg/ml) and gentamicin (20 μg/ml) under mineral oil at 38.5 °C in an atmosphere of 5% CO_2 for 45 min until addition of spermatozoa.

2.6. Sperm preparation and in vitro fertilization (IVF)

Frozen sperm samples from Andromed group and (A) group were thawed at 37 °C 21 s 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 $1500 \times g \times 5$ min. Supernatant was 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. 10 μl of this supernatant were taken and added to each fertilization droplet. The final sperm concentration was 1×10^6 sperm/ml in each droplet.

The sperm and oocytes were incubated for 24 h at 38.5 °C in a humidified 5% CO_2 atmosphere.

Zygotes were removed from fertilization medium and washed three times in embryo culture medium, SOF supplemented with 20 μl/ml essential minimum media BME, 10 μl/ml essential minimum media MEM, 5% (v/v) Fetal Bovine Serum, 54.75 μg/ml L-Glutamine, 8 mg/ml BSA and 40 μg/ml Gentamicin. Then, transferred to a 50 μL drops of the same medium under mineral oil, and cultured at 38.5 °C in 5% CO_2 in air for 48 h to evaluate fertilization parameters and for 4 days to evaluate embryo development.

2.7. Fertilization and embryo development rates

To evaluate *in vitro* embryo development rate (embryo development rate/fertilized oocytes) 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