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Long-term preservation of freeze-dried rabbit sperm by adding rosmarinic acid and different chelating agents

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ABSTRACT

Freeze-drying (FD) technique has been applied as an alternative technology to preserve gene resources to allow simple sperm preservation and shipment at 4 °C. Nevertheless, DNA sperm might be damaged by mechanical or oxidative stress throughout FD procedure. Therefore, suitable protection to maintain DNA integrity is required. The aim of this study was to determine the effect of rosmarinic acid (RA) as an antioxidant and two chelating agents (EGTA and EDTA) on the DNA integrity of freeze-dried rabbit sperm after storage of the samples at 4 °C and room temperature for 8 months. Rabbit sperm were freeze-dried in basic medium (10 mM Tris-HCl buffer and 50 mM NaCl) supplemented with 50 mM EGTA (1), 50 mM EGTA plus 105 μM RA (2), 50 mM EDTA (3) or 50 mM EDTA plus 105 μM RA (4). Semen samples were kept at 4 °C and room temperature during 8 months. After rehydration, DNA integrity was evaluated with Sperm Chromatin Dispersion test observing that DNA fragmentation was higher when semen samples were freeze-dried with EGTA (10.9%) than with EDTA (4.1%) ($p < 0.01$). Furthermore, RA acted better under adverse conditions and no significant differences were found in temperature storage. Summarizing, FD is a method that can allow simple gene resources preservation among 4 °C to 25 °C during 8 months and transportation without the need for liquid nitrogen or dry ice. EDTA chelating agent is the most suitable media for freeze-dried rabbit sperm and the addition of RA protects the DNA against the oxidative stress caused during FD procedure.

1. Introduction

Assisted Reproductive Technologies in mammals has been steadily progressing. Freeze-drying (FD) procedure, or lyophilisation, has been applied as an alternative technology to preserve gene resources [7–11,13,24]. The feasibility of this method is owing to the evidence that freeze-dried spermatozoa allows viable offspring by intracytoplasmic sperm injection (ICSI) in rabbits [15], mice [5,7,13,14,24,25], rats [3,4] and horses [1]. In addition, FD technology allows simple sperm preservation and shipment at 4 °C without noticeable deterioration, meaning that neither liquid nitrogen nor dry ice is required [4,7]. Room temperature would be ideal for long-term freeze-dried sperm storage and shipment.

Nevertheless, sperm DNA might be damaged by mechanical or oxidative stress throughout FD procedure [13,14]. It is suggested that Ca^{2+} and Mg^{2+} divalent cations activate sperm endogenous nucleases from plasma membrane during FD procedure causing DNA sperm damage and chromosome aberrations [13,21]. Previous studies in mice have reported that chelating agents such as ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA) or 2,2′,2″,2‴-(ethane-1,2-diyldinitrilo)tetraacetic acid (EDTA) added to freeze-drying medium can be combined with divalent cations forming stable complexes and thus protect DNA sperm from degradation [5,13,14]. Additionally, oxidative stress and reactive oxygen species (ROS) were previously associated with DNA sperm degeneration [13,14]. Rosmarinic acid (RA) is a natural antioxidant [20] that has shown therapeutic effects against oxidative stress in in vitro cell studies [12]. It has been demonstrated that the incorporation of RA in freezing [16,26] and FD [18] medium provides protection on spermatozoa against oxidative stress and thus improves the quality of sperm preservation.

To our knowledge, no studies have been performed to evaluate the effect of antioxidants on freeze-dried rabbit sperm.

The aim of this study was to determine the effect of RA as an antioxidant and two chelating agents (EGTA and EDTA) on the DNA integrity of freeze-dried rabbit sperm after storage at 4 °C and room temperature for 8 months. In addition, the influence of the storage temperature also was evaluated.

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2. Materials and methods

2.1. Reagents and media

Unless noted otherwise, all chemicals were from Sigma-Aldrich Co. (Alcobendas, Madrid, Spain). Freeze-drying control media used were: EGTA and EDTA. EGTA medium was composed of basic medium (10 mM Tris-HCL buffer and 50 mM NaCl) supplemented with 50 mM EGTA and EDTA medium was composed of basic medium (10 mM Tris-HCL buffer and 50 mM NaCl) supplemented with 50 mM EDTA. Two more experimental FD media were prepared by adding 105 μM RA to EGTA (EGTA-RA) and EDTA (EDTA-RA) [16]. The final pH of the solutions were adjusted to between 8.2 and 8.5 [9].

2.2. Animals

The study was performed following approval by the Veterinary Ethical Committee of University of Zaragoza. The care and use of animals were performed according to the Spanish Policy for Animal Protection RD1201/05, which meets the European Union Directive 86/609 on the protection of animals used for experimental and other scientific purposes.

All semen samples were collected from eight sexually mature bucks previously selected from at a commercial AI centre (Técnicas Cunícolas S.A.). Males were maintained in individual cages under light cycle of 12 h light/dark at a room temperature of 22–24 °C and a relative humidity of 55–60%. All animals were fed a commercial pellet diet according to their reproductive condition and fresh water was given ad libitum.

2.3. Collection of rabbit spermatozoa

Rabbit sperm samples were collected by artificial vagina. After semen collection, any gel plug was removed. Immediately a macroscopic analysis was performed to assess the colour and the volume of each ejaculate and the motility was evaluated by integrated sperm analysis system (ISAS®; PROISER R + D, Valencia, Spain). Only ejaculates with white colour, > 0.2 mL and good wave motion (at least 85% of motility) were used for the research. Thereafter all ejaculates were pooled, in order to eliminate individual differences, and diluted in pre-warmed (37 °C) INRA 96® (IMV Technologies, L'Aigle, France).

2.4. Freeze-drying and rehydration procedures

Freeze-drying procedure was performed as reported by Wakayama and Yanagimachi (1998) [24]. Heterospermic solution was divided into four falcon tubes for centrifuge at 700 g for 10 min at 37 °C and the supernatant was discarded. Afterwards, each falcon tube was resuspended in four FD media, EGTA, EGTA-RA, EDTA and EDTA-RA. 150 μL of sperm suspension from each group were placed into 1 mL volume glass cryovials (Labcon North, America, USA) and then plunged into liquid nitrogen (LN_2) for 5 min. Immediately the frozen samples were transferred onto the shelf (−50 °C) of a programmable freeze-drier (Lyobeta 25, Telstar). Two drying phases were performed for freeze-dry the samples: a primary drying at 0.053 mbar of pressure and at −68 °C and a second drying at 0.018 mbar of pressure and 20 °C of temperature. After FD process, cryovials were sealed with rubber cups and parafilm. Dried samples were stored in a conventional glass desiccator at 4 °C and room temperature (25 °C) during 8 months.

The rehydration of freeze-dried spermatozoa was performed by adding 300 μL of Milli-Q water. Rehydrated spermatozoa were centrifuged once at 1000*g* during 2 min and the supernatant was removed. The pellet was resuspended in 500 μL phosphate buffered saline (PBS) and thereafter the analysis of DNA integrity was performed by Sperm Chromatin Dispersion (SCD) test (Halotech DNA SL, Madrid, Spain).

2.5. Sperm DNA fragmentation analysis

Sperm Chromatin Dispersion test specifically designed for oryctolagus cuniculus spermatozoa (O.cuniculus-Halomax® kit) was used to evaluate sperm DNA fragmentation from freeze-dried spermatozoa. Succinctly, following the manufacturer's instructions, 25 μL of each diluted sperm sample was gently mixed with 50 μL of low melting point agarose. Subsequently, 2 μL of the cell suspension was placed onto marked wells and each drop was covered with 24 × 24 mm glass coverslip. The slides were kept horizontally at 4 °C for 5 min to solidify the agarose. Then coverslips were removed and the slides were fully immersed horizontally in 10 mL of lysis solution for 5 min. After washing the samples in distilled water for 5 min, the slides were dehydrated through two successive ethanol baths (70% and 100%) for 2 min in each one and air-dried. Finally, the cells were stained using a green fluorescence microscopy staining kit (FluoGreen®, Halotech DNA SL, Madrid, Spain). Once reagent A was mixed with reagent B in a 1:1 proportion, 3 μL of the mix was placed over the slide and was covered with a coverslip. The slides were checked under fluorescence microscopy (Olympus BX-40, Olympus U-RFL-T, Tokyo, Japan) at magnification 400× and at least 400 spermatozoa were counted per semen sample.

2.6. Statistical tests

The study was replicated three times. Data were analysed using IBM SPSS Statistics 23 for Windows. DNA fragmentation data were expressed in percentages and analysed by chi-squared test. The level of significance was set at $p < .050$.

3. Results

The different halo patterns of sperm DNA fragmentation after FD procedure analysed by SCD test are shown in Fig. 1. Spermatozoa with

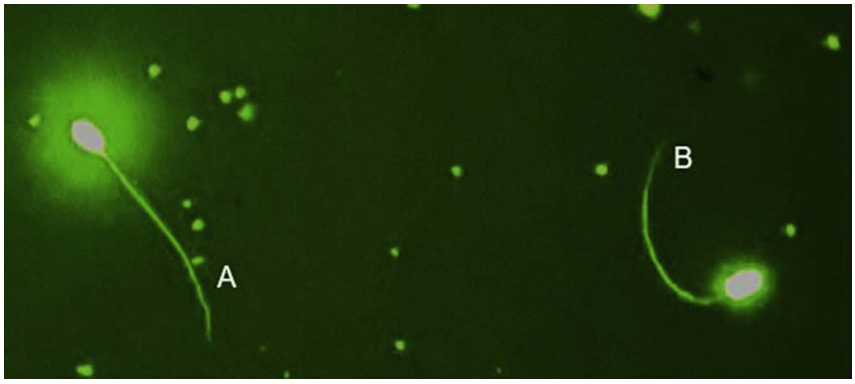

Fig. 1. Freeze-dried rabbit sperm processed with Sperm Chromatin Dispersion test. Fragmented sperm (A) and non-fragmented sperm (B).