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Original article

Chelating agents in combination with rosmarinic acid for boar sperm freeze-drying

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ABSTRACT

The presence of DNA protective agents in the medium is necessary to maintain sperm functionality after freeze-drying procedure. The objective of this study was to investigate the effect of chelating agents, ethylene diaminetetraacetic acid (EDTA) and ethylene glycoltetraacetic acid (EGTA), in combination with rosmarinic acid (RA) on DNA integrity of freeze-dried boar sperm. We also examined the effect of these agents on the *in vitro* developmental ability of porcine oocytes following sperm injection (ICSI). Heterospermic mix, obtained from ejaculated sperm of three boars, was freeze-dried in two different chelating agents' media: 50 mM EDTA or 50 mM EGTA, and in these media supplemented with 105 μM of rosmarinic acid. Frozen-thawed sperm was used as control. After rehydration, samples were subjected to DNA damage detection using Sperm Chromatin Dispersion test. ICSI was performed to verify the ability of freeze-dried sperm to participate in embryonic development. Five replicated trials were carried out for each group. In the presence of rosmarinic acid, the percentage of spermatozoa with DNA damage decreased significantly ($p = 0.010$), without differences between the two chelating agents combination. EDTA solution preserves more efficiently DNA integrity of boar sperm than EGTA solution ($p = 0.002$). There were no significant differences among the studied groups related to the blastocyst formation rate. Results suggested that the addition of rosmarinic acid to the medium improves sperm DNA integrity after freeze-drying, but does not promote fertilization and blastocyst development. We also observed a similar percentage of embryos production with freeze-dried and with frozen-thawed sperm.

1. Introduction

Cryopreservation is the most commonly used method to store boar spermatozoa for a long time, but using liquid nitrogen is associated with various inconveniences [1]. Recently, freeze-drying (FD) has been proposed as an alternative method for sperm preservation and genetic resources maintenance in different animal species [2]. The main advantage of the technique is the enormous cost reductions in storage and shipping for long-term preservation of sperm doses. Relative to conventional cryopreservation, FD is cheaper, uses no liquid nitrogen, requires less space for gamete storage, and it is an easier method for sperm transporting [3]. Although all the advantages mentioned above, FD causes more sperm damages than the conventional cryopreservation procedure. Kusakabe et al. [4] claimed that sperm DNA damage might be induced by the action of endonucleases or by oxidative stress during freezing and drying procedure and also, during the holding period before ICSI after rehydration. Besides, when mammalian spermatozoa are freeze-dried their motility is lost, so the intracytoplasmic sperm injection technique (ICSI) is necessary to fertilize oocytes [5–7]. A previous study on human sperm showed that the process of freeze-drying deeply damages cell membranes; however, unlike with liquid nitrogen preservation, it does not affect DNA integrity [8]. Therefore, the main requirement for freeze-dried sperm to produce offspring is the nucleus integrity. It has been shown that improved freeze-drying protocols preserve the chromosomal integrity and the oocyte-activating factors [9]. So it is important to identify the strategies to avoid or to reduce the sperm DNA damage during the FD process. It has been suggested that level of DNA fragmentation that

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occurred in the spermatozoa during FD depends on the solution used for treatment [10]. Kaneko and Nakagata [11] demonstrated that a small amount of a chelating agent in the FD solution is necessary to protect the sperm DNA from physical damage caused by the activity of endogenous nucleases. By that way, it is possible to prevent the deterioration of spermatozoa during the process and achieve the possibility of long-term preservation [7].

Sperm DNA damage during the FD process is also caused by oxidative stress [4]. Boar spermatozoa are particularly sensitive to the harmful effects of reactive oxygen species (ROS) due to the high content of polyunsaturated fatty acids in the plasma membrane and the low antioxidant capacity of seminal plasma [12,13]. The beneficial effect of antioxidant therapy on oxidative stress of cryopreserved mammalian spermatozoa has been previously reported [14–19]. Besides, the use of herbal antioxidants to preserve sperm cells has been studied during the last years [20–22]. Rosemary (*Rosmarinus officinalis*) aqueous extract is known to contain important scavengers of free radicals which may subsequently support the intracellular antioxidant system [23]. And, it has been successfully added into semen freezing extenders in several species, including swine [24], canine [25] and ovine [26]. Moreover, rosemary contains carnosic and rosmanic acids (RA) which play an important role in the antioxidant action of this plant [15]. The effect of RA has been studied on different animal cells, showing a beneficial antioxidant effect [27–29]. Luño et al. [30] suggested that RA is a potential candidate for use as an antioxidant in boar sperm cryopreservation extenders. To our knowledge, there are no studies focusing on determining the effect of antioxidant therapy on freeze dried boar sperm. Therefore the objective of this study was to examine the effect of ethylene diaminetetraacetic acid (EDTA) and ethylene glycoltetraacetic acid (EGTA) in combination with RA on DNA integrity of freeze-dried boar sperm, fertilization, and embryo development after intracytoplasmic sperm injection.

2. Materials and methods

The study was performed following approval by the Veterinary Ethical Committee of Universidad de 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.

2.1. Chemicals and media

Unless otherwise indicated, all chemicals were from Sigma-Aldrich Co. (Alcobendas, Madrid, Spain). The four freeze-drying media tested were: EGTA (10 mM Tris-HCl buffer + 50 mM NaCl + 50 mM EGTA), EDTA (10 mM Tris-HCl buffer + 50 mM NaCl + 50 mM EDTA), EGTAR (EGTA medium supplemented with 105 μM of aRA) and EDTAR (EDTA medium supplemented with 105 μM of RA). The pH of final solutions of each medium was adjusted to 8.0–8.2.

For the controls, the freezing medium was lactose-egg yolk (LEY) base extender (containing 20% (v:v) egg yolk, 11% of 80% L-lactose solution (290 mM), 100 μg/ml kanamycin sulfate).

The medium used for the collection of cumulus oocyte complexes (COCs) and washing was Dulbeco's phosphate buffered saline (DPBS) composed of 136.89 mM NaCl, 2.68 mM KCl, 8.1 mM Na_2HPO_4 and 1.46 mM $CaCl_2$ $2H_2O$ supplemented with 0.34 mM sodium pyruvate, 5.4 mM glucose, 70 μg/ml kanamycin and 4 mg/ml bovine serum albumin (BSA; fraction V). The basic medium used for oocyte maturation was a modified North Carolina State University medium (NCSU-23) [31] supplemented with 10% (v:v) porcine follicular fluid, 0.8 mM cysteine and 10 ng/ml epidermal growth factor (EGF), 10 IU/ml eCG and 10 IU/ml hCG.

ICSI was performed in 10 μl droplets of NCSU-23 supplemented with 0.4% (w:v) BSA (IVC 2) and 20 mM of Hepes under mineral oil. Two media were used for *in vitro* culture of sperm-injected oocytes: the first one was modified NCSU- 23 without glucose and supplemented with 0.17 mM sodium pyruvate, 2.73 mM sodium lactate, 4 mg/ml BSA (IVC 1), and the second one was IVC 2.

2.2. Sperm collection and freeze-drying procedures

The sperm-rich fraction was collected from three mature fertile Pietrain boars, aged between 2 and 3 years. The boars were housed individually in crates at a local boar stud. Semen samples were collected during winter-spring seasons, in a once a week seminal collection regime, by using the gloved-hand method. The semen was extended 1:2 in Beltsville Thawing Solution (BTS, Magapor, Spain) at approximately 15–20 °C for transportation. Sperm concentration, motility, and normal morphology were microscopically evaluated by standard laboratory techniques, and only high quality semen was selected for the experiments (motility >85%; total morphology abnormalities <15%). Once at the laboratory, semen doses were pooled and split into five equal fractions including control (cryopreserved) and four test groups (freeze-dried).

Cryopreservation of sperm was carried out using the straw freezing procedure, as described by Westendorf et al. [32] and modified by Carvajal et al. [33]. Sperm samples were centrifuged at 1500*g* for 3 min at 15 °C, the supernatant was discarded and the pellet was diluted in LEY base extender to yield a semen concentration of approximately 1500×10^6/ml. Sperm suspensions were gradually cooled from 15 to 5 °C over 2 h and then slowly diluted (2:1) with LEY extender containing 1.5% (v:v) OEP and 9% (v:v) glycerol. The final sperm concentration to be frozen was 1000×10^6/ml and 3% (v:v) glycerol. The diluted and cooled sperm were loaded into 0.5 ml straws and sealed. The sperm samples were frozen in static nitrogen vapor (4 cm above the liquid nitrogen for 20 min) and then plunged into liquid nitrogen for storage.

The fractions to freeze-dry were centrifuged at 600*g* for 3 min at 16 °C. The supernatant was discarded by aspiration and each fraction was resuspended in the four experimental FD media (EGTA, EGTAR, EDTA, and EDTAR) to a concentration of 150×10^6/ml and kept at 37 °C for 10 min. Then, 150 μl of sperm suspension from each group was placed into individual cryo tubes (Labcon North America, USA) and plunged into liquid nitrogen for 5 min. Samples were transferred to a programmable freeze-dryer (LyoBeta 25, Telstar, USA) previously cooled to −50 °C. Samples from the four treatments were submitted to the FD process under the same conditions: a primary drying at a pressure of 0.053 mbar and temperature of −68 °C, and a secondary drying at a pressure of 0.018 mbar and a temperature of 20 °C. The vials were stored at 4 °C for one year until used for DNA evaluation and ICSI.

2.3. Sperm preparation and DNA fragmentation analyses

Frozen samples were thawed by immersing in a circulating water bath at 37 °C for 21 s and resuspended in BTS (1:4, v:v; 37 °C) for DNA fragmentation analysis. Freeze-dried sperm samples were rehydrated by adding 150 μl Milli-Q water. The sperm suspension was centrifuged at 600*g* for 2 min and the supernatant was removed. The sperm pellet was resuspended in 500 μl phosphate buffered saline (PBS) for DNA fragmentation analysis.

Sperm from different treatments was evaluated by the Sperm Chromatin Dispersion test (SCD) specifically designed for boar spermatozoa (Sperm-Sus-Halomax®) following the manufacturer's instructions. All the samples were stained using a commercial kit for green fluorescence staining (Fluogreen, Halotech DNA SL, Spain) and evaluated using fluorescent