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α-Tocopherol modifies tyrosine phosphorylation and capacitation-like state of cryopreserved porcine sperm

M.M. Satorre, E. Breininger, M.T. Beconi, N.B. Beorlegui *

Area of Biochemistry, School of Veterinary Sciences, University of Buenos Aires, Chorroarín 280, C1427CWO Buenos Aires, Argentina

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Abstract

Sperm cryopreservation is associated with the production of reactive oxygen species (ROS) leading to membrane destabilization, which induces capacitation-like changes, increases protein tyrosine phosphorylation, and decreases their fertilizing ability. α-Tocopherol, a lipid peroxidation inhibitor, preserves the functionality of cryopreserved porcine sperm. Our aim was to evaluate the effect of α-tocopherol on sperm quality parameters as well as capacitation-like changes and modifications in protein tyrosine phosphorylation. Boar sperm frozen with or without 200 μg/mL of α-tocopherol were thawed and maintained at 37 °C for 10 min in BTS. Routine parameters of semen quality were evaluated by optical microscopy and membrane changes were determined by the epifluorescence chlortetracycline technique. Changes in protein tyrosine phosphorylation were examined using a specific anti-phosphotyrosine monoclonal antibody. Motility was higher (18%, $P < 0.05$) in semen with α-tocopherol. Viability did not differ ($P > 0.05$) between treatments. However, there was less ($P < 0.05$) capacitation-like changes in semen with α-tocopherol compared to control samples. A MW 32 kDa tyrosine-phosphorylated protein was detected in extracts of cryopreserved sperm; the intensity of immunostaining was lower in semen containing α-tocopherol compared to the control (0.211 ± 0.030 versus 0.441 ± 0.034 arbitrary units). Additionally, this band was not detected in fresh sperm. The addition of α-tocopherol to the extender prior to cryopreservation of boar semen protected sperm membranes against oxidative damage and reduced both tyrosine phosphorylation and the capacitation-like state.

Keywords: Cryopreservation; Boar sperm; Antioxidants; Capacitation-like changes; Tyrosine phosphorylation

1. Introduction

Semen cryopreservation includes dilution, cooling, freezing, and thawing that alter sperm plasma membrane [1]. Freezing-thawing alters selective permeability [2], decreases energy production [3], and reduces sperm motility [4]. The membrane destabilization may be related to the lipid composition of the bilayer, affecting its fluidity and making it more permeable, thus allowing free calcium to enter the cell, which stimulates capacitation-like changes (cryocapacitation) [5]. In cattle sperm, cryocapacitation was induced in a non-regulated manner, independent of internal pH, and mediated by a different mechanism than *in vitro* capacitation by heparin [6]. In the boar, it has been postulated that cryopreserved sperm undergo alterations similar to those occurring during capacitation [7–9]. Depending on the method used to evaluate capacitation, these changes may or may not be detected. In that regard, although freezing-thawing did not increase cryocapacitated sperm (based on the Merocyanine-540

* Corresponding author. Tel.: +54 11 4524 8452; fax: +54 11 4524 8452.
E-mail address: nbeorlegui@fvet.uba.ar (N.B. Beorlegui).

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assay [10,11], changes in the chlortetracycline pattern and in intracellular free calcium were similar to those observed in true sperm capacitation [11].

Moderate cooling of sperm (to 10 °C) accelerated capacitation behavior in terms of the response to ionophore [12]. Sperm undergoing capacitation-like changes could undergo premature spontaneous acrosome reactions and lose their ability to fertilize [9]. In these destabilized cells, when plasma membrane modifications and the increase in intracellular calcium occurred too rapidly, sperm capacitation soon followed; therefore, sperm membranes degenerated before fusion with the oocyte [13]. Thus, the lifespan of frozen-thawed sperm in the female reproductive tract was considerably shorter than that of freshly ejaculated sperm [9,14].

Protein phosphorylation is a post-translational modification that allows the cell to control various cellular processes. Mature sperm are highly compartmentalized, transcriptionally inactive, and unable to synthesize new proteins. Therefore, protein phosphorylation is an important means of modifying sperm function. In various species, including mice [15], human [16], cattle [17] and swine [18], it has been well documented that capacitation appears to be associated with tyrosine phosphorylation of sperm proteins. In the boar, tyrosine phosphorylation of one or more proteins with molecular masses of 32 kDa could be associated with capacitation [19].

Reactive oxygen species (ROS) are active molecules with a dual role in sperm metabolism, depending on the amount produced. At physiological concentrations, ROS participated in normal sperm function, but an excess of ROS produced oxidative stress [20,21]. Freezing and thawing increased ROS generation [22]. Boar sperm are extremely sensitive to peroxidative damage, due to the high content of unsaturated fatty acids in the phospholipids of the plasma membrane [1]. The addition of natural antioxidants such as α-tocopherol had a protective effect on the plasma membrane of cryopreserved bovine sperm, preserving both metabolic activity and cellular viability [23,24]. Inclusion of α-tocopherol (200 μg/mL) in the freezing extender prevented oxidative damage and improved sperm motility in cryopreserved boar semen [25].

Use of cryopreserved boar semen is restricted since it has lower fertility than fresh semen [26]. With current protocols for cryopreserving boar sperm, including the Beltsville [27] and the Hülsenberg [28] methods, post-thaw survival was restricted to approximately 50% of the sperm population [7]. Therefore, it is desirable to improve the functional capacity of cryopreserved sperm.

The objective of the present study was to determine if the presence of α-tocopherol in the cryopreservation extender would affect capacitation-like changes and tyrosine-phosphorylated sperm proteins in cryopreserved porcine sperm.

2. Materials and methods

2.1. Chemical reagents

Unless otherwise indicated, reagents were obtained from Sigma Chemical Company (St. Louis, MO, USA). Orvus ES Paste (Equex-Paste) was from Minitub (Tiefenbach b. Landshut, Germany), Trypan Blue, Eosin Y, and Nigrosine were from Mallinckrodt (St. Louis, MO, USA) and Red Ponceau was from Stanton, Buenos Aires, Argentina. Dextrose, sodium citrate, sodium bicarbonate, sodium chloride, EDTA, potassium chloride, disodium hydrogen phosphate, and potassium dihydrogen phosphate were from Merck (Darmstadt, Germany).

2.2. Semen collection

Samples were collected (gloved-hand technique) from four crossbred boars (Pietrain × Yorkshire, 1–1.5 years old) of proven fertility. These boars belonged to an AI program and were kept under uniform feeding and handling conditions throughout the study. Only semen samples with a minimum of 70% motile and 80% morphologically normal sperm were used.

2.3. Semen cryopreservation

Boar semen was cryopreserved according to Pursel and Johnson's protocol, as described by Breininger et al. [25]. After a holding period of 2 h at 20 °C, each semen sample was split into two fractions and centrifuged for 10 min at 300 × *g*, and the seminal plasma was removed by aspiration. One fraction was resuspended in Beltsville F5 extender (BF5) [25] without α-tocopherol (control samples), whereas the other fraction was resuspended in BF5 containing 200 μg/mL of α-tocopherol acetate (concentration of 2×10^8 sperm/mL in both fractions). Sperm suspensions were cooled to 5 °C over 2 h and mixed with an equal volume of BF5 with 2% glycerol (final concentration, 1×10^8 sperm/mL). The contents were frozen (in 0.1 mL pellets) in dry ice (−76 °C) and stored in liquid nitrogen (−196 °C).

2.4. Preparation of sperm suspension

Pellets were placed into a conical centrifuge tube containing Beltsville thawing solution (BTS, 1:1 [pellet:mL]) [25] and mixed gently. Fresh semen was