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Boar sperm protein tyrosine phosphorylation in the presence of egg yolk soluble and low density lipoprotein fractions during cooling

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ABSTRACT

Increased protein tyrosine phosphorylation and the appearance of a phosphorylated protein of 32 kD (p32) are reported among the capacitation-like changes in cryopreserved boar sperm. Egg yolk freezing extenders are composed by two fractions: insoluble granules and soluble plasma, which contains the low density lipoproteins (LDL) proposed as responsible for the egg yolk cryoprotective action. The aim of this work was to analyze the effects of complete egg yolk and its insoluble, soluble and LDL fractions on boar sperm quality and protein tyrosine phosphorylation after the first stage of a standard cryopreservation protocol. Semen samples in Androstar® Plus diluent were centrifuged and resuspended in the different egg yolk extenders. Temperature was decreased from 17 °C to 5 °C and sperm quality, protein tyrosine phosphorylation and protein pattern were analyzed. Results showed that complete egg yolk as well as soluble and LDL egg yolk fractions maintained sperm quality after temperature decrease. Cooling without any lipid component or in the presence of the insoluble fraction, significantly reduced sperm motility. About sperm protein tyrosine phosphorylation analysis, the p32 band appeared before treatments or after cooling in Androstar® Plus diluent. Complete egg yolk and its insoluble fraction interfered with sperm tyrosine phosphorylation even after cells were extensively washed. Analysis of extenders revealed a high amount of tyrosine phosphorylated proteins in the insoluble fraction, which may have co-precipitate with sperm in experiments. Samples submitted to temperature decrease from 17 °C to 5 °C in the presence of soluble and LDL egg yolk fractions in Androstar® Plus diluent did not show any change in the p32 band associated with sperm capacitation. However, a tyrosine-phosphorylated protein of 33 kD present in clarified egg yolk was also observed in sperm treated with this extender. Protein transference from plasma and LDL egg yolk extenders was also observed in sperm protein profile. Results suggested that soluble and LDL fractions might have a protective action preventing sperm protein tyrosine phosphorylation during cooling from 17 °C to 5 °C. Further studies are needed to expand the knowledge of the LDL protection mechanism as well as to determine the possible benefits of clarified egg yolk in freezing protocols.

1. Introduction

Cryopreservation or so-called freezing of sperm allows samples to be stored indefinitely for genetic banking or distributed for artificial insemination programs. However, a significant number of sperm do not survive the freezing and thawing process and those that do may exhibit premature capacitation-like changes, sometimes called cryocapacitation, that reduce their functional ability and subsequent fertility results. Frozen thawed sperm may exhibit premature acrosome reaction since cryocapacitation resembles true capacitation events in many ways. Therefore, sperm would present a very short half-life in the female genital tract. Moreover,

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membrane destabilization may lead to an increase in its permeability, membrane disruption and cell death [1–7].

Boar spermatozoa are particularly susceptible to temperature decline. Increased protein tyrosine phosphorylation and the appearance of a phosphorylated protein of 32 kD (p32) were reported among the capacitation-like events. However, those results are controversial and several authors conclude that protein phosphorylation pattern depends on the preservation procedure, the individual animal and the sample [2,3,8–14].

It is widely known that cholesterol is one of the factors that determine the biophysical properties of biological membranes and, therefore, regulates conformational changes and function of membrane proteins [15]. Since sperm are highly differentiated cells which have lost most of their synthesis machinery, membrane cholesterol content is regulated depending on the cholesterol concentration of the environment. During physiological sperm capacitation, cholesterol efflux takes place and the plasma membrane becomes more fluid [16,17]. Boar sperm plasma membrane presents a low cholesterol/phospholipid ratio (0.26) and a high content of unsaturated phospholipids, which leads to a high fluidity membrane and a short capacitation period [18,19]. Similarly, it is thought that the response to low temperature is strongly related to the lipid composition of the membrane.

In most of freezing protocols, seminal plasma is removed and sperm are mixed with cryoprotectant media called extenders [6,7]. These media include lipids to stabilize the cell membrane, hen egg yolk being the most widely used lipid source. Equex-Paste®, a commercial surfactant, which emulsifies and disperses lipids, is usually added to egg yolk extenders since it has been reported that it decreases freeze-thaw damage and optimizes post-thaw motility, acrosome morphology and fertilization rates [20]. Other protocols include the use of cholesterol or cyclodextrins loaded with cholesterol [21–23]. Several protocols use egg yolk low density lipoproteins (LDL) for cryopreserving sperm of different species [24–31], because this egg yolk fraction has been proposed as responsible for the cryoprotective action [32–34].

Egg yolk is a lipid-in-water emulsion with about 65% lipids, 31% proteins and 4% carbohydrates, vitamins and minerals, and it is composed by two fractions, an insoluble granule fraction and a soluble plasma fraction. The main components of egg yolk are 68% LDL, 16% HDL, 10% livetins and 4% phosvitins. Plasma contains 85% LDL and 15% livetins, being β-livetin a tyrosine phosphorylated protein of 33 kD. Granules consist of 70% HDL, 12% LDL, which is very similar to the plasma LDL, and 16% phosvitin, the principal phosphoprotein of egg yolk [35,36]. It has been reported that complete egg yolk has several deleterious components to sperm and that its progesterone content might induce sperm acrosome reaction [37]. Furthermore, Tosic et al. [38] observed that granules reduce respiration and motility in bovine spermatozoa.

Most research groups use complete egg yolk as non-permeable cryoprotectant and evaluate the effect of different experimental variables such as holding time, cooling rate, concentration of permeable cryoprotectants and Equex-Paste®, presence of different antioxidants, etc. [6,7,11,39–44]. In the same way, several studies have analyzed the effect of LDL on sperm motility, vitality and DNA in different species with variable results [24–30]. However, LDL effects on protein tyrosine phosphorylation associated with cryocapacitation have never been studied. Moreover, only a few studies have reported the effect of the soluble and insoluble fractions of the yolk in sperm cryopreservation [32,34,45].

The aim of this work was to analyze the effects of complete egg yolk and its insoluble, soluble and LDL fractions on boar sperm quality and protein tyrosine phosphorylation after the first stage of a standard cryopreservation protocol.

2. Materials and methods

2.1. Chemicals

All reagents used were of high purity or analytical grade and purchased from Sigma Chemical Co. (St. Louis, MO, USA), Fisher Scientific (Loughborough, Leicester, UK), Merck (Darmstadt, Hesse, Germany) or J.T.Baker (Phillipsburg, NJ, USA).

2.2. Samples

Semen samples were obtained by the standard gloved-hand technique from five adult hybrid boars (cross of three pure breeds: Large White, Pietrain and Hampshire) housed at an artificial insemination center in the School of Veterinary Sciences of the University of Buenos Aires. Handling of animals was in accordance with the principles expressed in the "Legislation for the protection of animals used for scientific purposes" (European Commission).

Pre-and post-sperm-rich fractions were discarded, and the sperm-rich fraction was used for analysis. The following parameters were measured to determine semen quality: ejaculate volume and sperm viability, motility, concentration, morphology, and response in the hypo-osmotic swelling test (HOST). Only samples which met these quality requirements were used: volume greater than 50 mL, progressive motility greater than 70%, abnormal sperm below 20%, and concentration of at least 3×10^8 sperm/mL. Ejaculates were processed individually.

2.3. Extenders preparation

Yolks from fresh commercial eggs were manually separated from the albumen and carefully rolled on an absorbing paper to remove albumen adhering to vitelline membrane and chalazas. The vitelline membrane was broken and the yolk was collected. The following extenders were prepared: 20% egg yolk in 11% lactose or in Androstar® Plus diluent (Minitube, GmbH), depending on the experiment; soluble, insoluble and LDL egg yolk fractions in the same diluent. For soluble and insoluble fractions, 20% egg yolk in the respective diluent was centrifuged at 10,000 × g for 45 min at 4 °C. For soluble fraction, the supernatant was centrifuged again in the same conditions for complete removal of granules. For insoluble fraction, both precipitates were pooled, washed twice with Androstar® Plus diluent by centrifugation at 10,000 × g at 4 °C for 45 min and resuspended to initial volume in the appropriate diluent.

LDL was obtained according to the method of Moussa et al. [24]. Briefly, yolk was diluted 1:1 with isotonic saline solution (0.17 M NaCl), stirred for 1 h and the supernatant (plasma) was obtained by two consecutive centrifugations at 10,000 × g for 45 min at 10 °C. Plasma was mixed with 40% ammonium sulfate for 1 h to precipitate livetins. The pH of the plasma was fixed and controlled at 8.7 and the temperature was fixed at 4 °C. By centrifugation at 10,000 × g at 4 °C for 45 min, the supernatant was separated from the sediment. The supernatant was then dialyzed against distilled water until complete ammonium sulfate elimination and centrifuged (10,000 × g for 45 min). The floating residue, rich in LDL, was collected, aliquoted and conserved at −20 °C. Preparations of 20% LDL in Androstar® Plus diluent were used for experiments.

2.4. Equex effect

To evaluate the capability of Equex-Paste® to solubilize egg yolk components, precipitate obtained with different concentrations of this surfactant were weighed. Briefly, increasing amounts of Equex-Paste® (Minitube, GmbH) were added to 20% egg yolk preparation