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An evaluation of soybean lecithin as an alternative to avian egg yolk in the cryopreservation of fish sperm ☆

Cengiz Yildiz [a,*], Yusuf Bozkurt [b], Ilker Yavas [a]

[a] Department of Reproduction and Artificial Insemination, Faculty of Veterinary Medicine, University of Mustafa Kemal, Hatay, Turkey
[b] Department of Aquaculture, Faculty of Fisheries, University of Mustafa Kemal, Hatay, Turkey

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ABSTRACT

Plant-derived lecithin has been used as a more sanitary alternative to avian egg yolk in livestock sperm cryopreservation protocols but its efficacy for cryopreserving fish sperm has not previously been tested comparatively. Here various concentrations of soybean lecithin were evaluated for the cryopreservation of carp (*Cyprinus carpio*) sperm. Sexually mature fish were induced to spermiation and ovulation with ovopel. The extenders were prepared by using 300 mM glucose, 10% DMSO, supplemented with different ratios of lecithin (5%, 10%, 15%, and 20%) and 10% egg yolk (control I). Negative control was made without egg yolk and soybean lecithin (control II). The pooled semen was diluted separately at ratio of 1:3 (v/v) by using egg yolk and soybean-based extenders. Diluted semen placed into 0.25 ml straws were equilibrated at 4 °C for 15 min and frozen in liquid nitrogen vapor. Fertilization was conducted using a ratio of 1×10^5 spermatozoa/egg. Supplementation of 10% lecithin to extender showed the best cryoprotective effect for sperm motility and duration of motility against freezing damage compared to 15%, 20% and control II groups ($p < 0.05$). Cryopreserved sperm with extender containing 10% lecithin provided a greater result in terms of fertilization success when compared to extenders containing 20% lecithin or control II ($p < 0.05$).

It is concluded that the animal protein-free extender containing 10% soybean lecithin has a similar cryoprotective actions with conventional egg yolk-based extender against freezing damages and fertilization. Therefore, soybean lecithin is a suitable alternative to avian egg yolk for the cryopreservation of fish sperm.

Introduction

Long-term storage of sperm in liquid nitrogen is a valuable technique for genetic resources preservation [21,36]. Research on fish sperm cryopreservation has achieved great advances since the first successful sperm cryopreservation in herring [12]. It provides many benefits such as ease of global germplasm shipping and supply [31], selective breeding and hybridization with desirable characteristics and also conservation of genetic diversity [23]. Furthermore, cryopreservation is considered as one component in an effective strategy to save endangered species by facilitating the storage of their gametes in gene banks [8,21].

Even though many successes have been achieved in fish semen cryopreservation, the technique remains as a method that is difficult to be standardized and use in all types of fishes. This is due to the fact that cryopreservation of sperm from different fish species required different conditions, where the protocol needs to be established individually. Cryopreservation techniques involve addition of cryoprotectants, freezing and thawing of sperm samples, all of which may result in some damage to the spermatozoa and may decrease egg fertilization success [19]. Therefore, before cryopreservation of sperm, a thorough evaluation of different extender solutions, cryoprotectants, and thawing rates is essential to develop optimum cryopreservation protocols for various fish species [35].

An extender should contain an energy source substrate (i.e., glucose or fructose), a source of lipoprotein or high-molecular-weight material to prevent cold shock (such as egg yolk, milk or soybean lecithin), ionic or nonionic substances to maintain a suitable osmotic pressure and pH, and other additives, such as enzymes and antibiotics [1,33]. Extender solutions contain egg yolk due to the low-density lipoprotein fraction like lecithin, which protects the membrane phospholipid integrity during cryopreservation [6,22]. However, the use of egg yolk has associated sanitary risks, including the production of harmful metabolites and toxins and the risk of infection, all resulting in reduced semen quality [2,5,13]. In fact, the risk of introducing exotic diseases (i.e., avian influenza)

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* Corresponding author. Fax: +90 326 245 5704.
E-mail address: cyildiz@mku.edu.tr (C. Yildiz).

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through the transportation of egg-based products is a widespread concern [13]. Therefore, a well-defined and pathogen-free substitute of non-animal origin for egg yolk would be preferable. It has been suggested that replacing egg yolk as the non-permeable cryoprotectant in cryogenic extenders with plant-based LDLs, such as soybean lecithin, may reduce sanitary risks and improve cryopreserved semen quality and fertility [3,5,9,18]. Soybean lecithin is currently used in commercially prepared extenders such as viz Andro-Med (Andromed, Minitube, Germany), Biociphos plus, and Bioxcel l [1,7,22,18], which have been tested and used successfully for the cryopreservation of bovine, caprine and ovine semen. To the best of our knowledge, the present paper document is the first comparative research related with soybean lecithin as an alternative to egg yolk for the cryopreservation of fish spermatozoa. Therefore, this study was designed to compare the effect of various levels of soybean lecithin in extender on quality (motility, duration of motility, and viability) and fertility of cryopreserved carp sperm.

Materials and methods

Broodstock management

Carp broodstock were collected from wintering ponds by seining and transported into the hatchery 48 h prior to gamete collection. In the hatchery, male (2460.4 ± 25.2 g; $n = 10$) and female (3520.5 ± 48.6 g; $n = 6$) broodfish were held separately in shadowed tanks ($V = 1000$ L) supplied with continuously (2.5 L min^{-1}) well-aerated water of 24 °C.

Gamete collection

Broodstock were anesthetized in 100 ppm of MS 222 (Tricaine methanesulfonate; Argent Labs., Redmond, WA, USA). Sperm was collected from ten males by manual abdominal stripping 12 h after a single injection of 2 mg/kg of CPE (Carp pituitary extract; Argent Labs., Redmond, WA, USA) at 24 °C water temperature. For sperm collection, the urogenital papilla's of mature male fishes were carefully dried and sperm was hand-stripped directly into test tubes. Tubes containing sperm were placed in a styrofoam box containing crushed ice (4 °C). Contamination with water, urine or feces of sperm was carefully avoided.

Eggs were collected by hand stripping 10–12 h after a double injection of 3.5 mg/kg of CPE. The first injection that is 10% of the total amount (0.35 mg/kg), was given 10 h before from the second (3.15 mg/kg) injection of CPE (14). Their abdomens and urogenital papilla were dried before stripping. Samples contaminated with fecal material or urine was discarded. Only transparent, well rounded and unwrinkle eggs were used for fertilization. Eggs were collected by hand stripping 10–12 h after a double injections of 3.5 mg/kg of CPE. The first injection that is 10% of the total amount (0.35 mg/kg), was given 10 h before the second (3.15 mg/kg) injection of CPE (14).

Sperm quality

Sperm motility was estimated subjectively using light microscope (Olympus, Japan) with a ×400 magnification. Samples were activated by mixing 1 μl of sperm with 20 μl activation solution (0.3% NaCl) on a glass slide. The motility percentages were defined as the percentage of spermatozoa moving in a forward motion every 20% motile increment (i.e., 0, 20%, 40%, 60%, 80%, and 100%) [34]. Motility measurements were performed within 15 s following sperm activation. Sperm cells that vibrated in place were not considered to be motile. For cryopreservation experiments, sperm samples showing below 80% motility were discarded. Following addition of the activation solution to the sperm samples, duration of sperm motility was determined using a sensitive chronometer (sensitivity: 1/100 s) by recording the time until all spermatozoa movements have stopped. Approximately twenty spermatozoa were counted for each examination area on a glass slide and totally sixty spermatozoa were observed by using three different evaluation areas for same straw.

Sperm density was determined according to the haemacytometric method [30]. Sperm was diluted at ratio of 1:1000 with Hayem solution (5 g Na_2SO_4, 1 g NaCl, 0.5 g $HgCl_2$, 200 mL bicine) and density was determined using a 100 μm deep Thoma haemocytometer (TH-100, Hecht-Assistent, Sondheim, Germany) at 400× magnification with Olympus BX50 phase contrast microscope (Olympus, Japan) and expressed as spermatozoa $\times 10^9$ mL^{-1} (three replicates). Counting chambers were always kept in a moist atmosphere for at least 10 min before cell counting. Sperm pH was measured using indicator papers (Merck, 5.5–9) within 30 min of sampling. To assess live/dead sperm percentage, eosin-nigrosin preparations were made according to the method noted by Björndahl et al. [11]. A total of 300 sperm cells were counted on each slide at 1000× magnification.

Sperm dilution

The semen extender containing 300 mM glucose, 10% dimethyl sulfoxide (DMSO), 10% egg yolk in 100 ml distilled water was prepared according to Bozkurt et al. [14] as the basic extender (egg yolk-based extender /reference control I). Extenders containing soybean lecithin were mixed in a water bath at 50 °C until completely dissolved. The extender without egg yolk was diluted separately using either 5%, 10%, 15%, or 20% soybean lecithin (P5638, Sigma–Aldrich, St. Louis, MO, USA) as experimental groups. Negative control (control II) was made without egg yolk and soybean lecithin.

Adjusted and pooled semen according to same sperm concentrations from ten males was diluted at ratio of 1:3 (v/v) with the experiment groups at 30 °C. Collected semen from males that showing >80 motility was pooled into equal aliquots according to the required semen volume and sperm density needed to eliminate effects of individual variability of the donors.

Cryopreservation procedure

The diluted samples were drawn into 0.25 ml plastic straws (IMV, France) and were sealed with polyvinyl alcohol (PVA). Having been diluted, the samples were equilibrated for 15 min at 4 °C in the plastic straws. After equilibration, the straws were placed on a styrofoam rack that floated on the surface of liquid nitrogen in a styrofoam box. The straws were frozen in liquid nitrogen vapor 3 cm above the surface of liquid nitrogen (−120 °C) for 10 min (30). Following, the straws were plunged into the liquid nitrogen (−196 °C) and stored for several days. Frozen straws were thawed in waterbath of 35 °C and for 30 s whereby straws were gently agitated during thawing. Thawed sperm was activated using 0.3% NaCl and observed under microscope for determination of motility and motility durations.

Fertilization experiment

For fertilization, pooled eggs from six mature females were used to determine fertilization rates. Egg samples (about 1000 eggs) were inseminated in dry Petri dishes with fresh sperm or frozen sperm immediately after thawing at spermatozoa:egg ratio of 1×10^5:1 (14). Thawed sperm was added over the eggs and gently mixed before activation with 20 ml of fertilization solution (3 g urea and 4 g NaCl in 1 L distilled water). After fertilization the eggs

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