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Fertility and flow cytometric evaluations of frozen-thawed rooster semen in cryopreservation medium containing low-density lipoprotein

A. Shahverdi [a,*], M. Sharafi [a,b,*], H. Gourabi [c], A. Amiri Yekta [c], V. Esmaeili [a], M. Sharbatoghli [a], E. Janzamin [d], M. Hajnasrollahi [e], F. Mostafayi [e]

[a] Department of Embryology at Reproductive Biomedicine Research Center, Royan Institute for Reproductive Biomedicine, ACECR, Tehran, Iran
[b] Department of Poultry Science, Faculty of Agriculture, Tarbiat Modares University, Tehran, Iran
[c] Department of Genetics at Reproductive Biomedicine Research Center, Royan Institute for Reproductive Biomedicine, ACECR, Tehran, Iran
[d] Department of Stem Cell and Developmental Biology at Cell Science Research Center, Royan Institute for Stem Cell Biology and Technology, ACECR, Tehran, Iran
[e] Animal Core Facility at Reproductive Biomedicine Research Center, Royan Institute for Biotechnology, ACECR, Tehran, Iran

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ABSTRACT

Frozen-thawed rooster semen is not reliable for use in artificial insemination in commercial stocks. Low-density lipoprotein (LDL) has been assessed for effectiveness as a cryoprotectant in the extender to improve the quality of frozen-thawed rooster semen. Although LDL has been evaluated in a few studies in other species for semen cryopreservation, so far no study has been conducted to examine this cryoprotectant for cryopreservation of fowl semen. Thus, this study aims to analyze the effects of different concentrations of LDL (0%, 2%, 4%, 6%, and 8%) in a Beltsville extender for cryopreservation of rooster spermatozoa. In experiment 1, motion parameters, membrane integrity, acrosome integrity, apoptosis status, and mitochondria activity were assessed after freeze-thawing. The highest quality frozen-thawed semen was selected to be used for evaluation of the fertility rate in experiment 2. Semen was collected from six roosters, twice weekly, then extended in a Beltsville extender that contained different concentrations of LDL as follows: 0% (control), 1% (Beltsville plus 1% LDL [BLDL1]), 2% (BLDL2), 4% (BLDL4), 6% (BLDL6), and 8% (BLDL8). Supplementation of the Beltsville extender with 4% LDL produced the most significant percentage of motility (43.1 ± 1.3), membrane integrity (59.4 ± 2.1), mitochondria activity (49.1 ± 1.19), and viable spermatozoa (45 ± 2.28) compared with the control treatment with the results of 22.7 ± 1.3 (motility), 38.4 ± 2.1 (membrane integrity), 40.25 ± 1.19 (mitochondrial activity), and 37.8 ± 2.28 (viability). In experiment 2, a significantly higher percentage of fertility rate was observed for frozen-thawed semen in the extender supplemented with 4% LDL (49.5 ± 1.6) compared with the control (29.2 ± 2.9). Progressive motility and acrosome integrity were not affected by LDL levels in the extenders. The results revealed that supplementation of the Beltsville extender with 4% LDL resulted in higher quality of frozen-thawed rooster sperm.

* Corresponding authors. Tel.: +98 21 23562674; fax: +98 21 23562677.
E-mail addresses: shahverdi@royaninstitute.org (A. Shahverdi), m.sharafi@royaninstitute.org (M. Sharafi).

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1. Introduction

Although numerous attempts have been made to develop a suitable procedure for cooled preservation of rooster semen, a successful protocol for cryopreservation has not been achieved [1–4]. The lower fertility rate of cryopreserved poultry semen compared with other species is a serious challenge for artificial insemination in commercial flock [5,6]. This challenge may be related to some special physiological characteristics of poultry semen, which increase their susceptibility to damage during cryopreservation [7,8]. During freeze-thawing, rooster spermatozoa are exposed to cryoinjuries that result from the sudden cold and warm shock, which leads to membrane rupture, reduction in semen quality, and fertilization potential [9]. Therefore, developing a suitable cryopreservation medium is crucial for semen integrity after freeze-thawing. The most common extender for cryopreservation of rooster semen is the Beltsville extender, which produces highly variable results because of the lack of optimization of the extender [10,11]. The Beltsville extender is composed of dipotassium phosphate, sodium glutamate, fructose, and sodium acetate in addition to other buffers and salts. Therefore, the addition of an extracellular cryoprotectant for the protection of spermatozoa against the stress of freezing (reactive oxygen species) seems to be crucial. It has been proposed that low-density lipoprotein (LDL) from egg yolk is a critical cryoprotectant, which is mainly responsible for resistance against cryopreservation stress assisting with preservation of the quality of frozen-thawed semen [12–15]. It has been reported that LDL by its attachment to the cell membrane and small proteins in the seminal plasma can preserve sperm quality after cryopreservation [16,17]. Low-density lipoprotein contains approximately 20% phospholipids, which are considered to be the effective part of the LDL in the extenders [18]. Therefore, it seems that this extracellular cryoprotectant is a good choice for improving the Beltsville extender and producing higher quality semen after freeze-thawing.

The objective of this study was to modify the Beltsville extender by evaluating different concentrations of LDL and comparing them with the control extender. Two experiments were conducted after the freeze-thawing process. In the first experiment (experiment 1), semen quality parameters such as motion characteristics, membrane integrity, acrosome integrity, apoptosis features, and mitochondrial activity were assessed followed by cryopreservation. The second experiment (experiment 2) was performed based on artificial insemination for comparing the fertility rate for the Beltsville extender and the best level of LDL obtained in experiment 1.

2. Materials and methods

2.1. Chemicals

All chemicals used in this study were purchased from Sigma (St. Louis, MO, USA) and Merck (Darmstadt, Germany). This study received the approval of the Ethics Committee of Royan Institute.

2.2. Farm management and semen collection

This research was conducted at the Department of Embryology at Royan Institute. Six mature white leghorn layer breeder roosters were housed individually in cages ($70 \times 70 \times 85$ cm) at 18 °C to 22 °C, under a 15 hours light and 9 hours dark photoperiod. Animals were fed a diet that contained 10% crude protein, 3170 kcal maintenance energy/kg, 0.9% calcium, and 0.45% available phosphate.

For artificial insemination, 20 white leghorn hens were kept individually in conditions similar to the roosters. All the roosters and hens were 30 weeks at the beginning of the experiment. Semen was collected weekly from the six roosters by a method described by Burrows and Quinn [19]. Immediately after collection, the ejaculates were transferred to a water bath (37 °C) and then evaluated for primary criteria such as volume, color, morphology, and concentration. Ejaculates would be accepted to be used in this study if they met the following conditions: volume of 0.2 to 0.6 mL, concentration of 3×10^9 spermatozoa/mL or greater, motility of 80% or greater, and abnormal morphology of 10% or less. To eliminate individual differences and obtain sufficient sperm for analysis, we pooled the semen and subsequently divided them into six aliquots.

2.3. Low-density lipoprotein extraction from egg yolk

Low-density lipoprotein was extracted from egg yolk according to the method described by Moussa et al. [16] with few modifications. Fresh eggs were collected from one flock of hens that received the same standard diet. After disinfection with 75% ethanol, eggs were manually broken and yolks separated from the albumen. All egg yolks were carefully rolled on a filter paper to remove chalazae and traces of albumen that adhered to the vitellin membrane. The vitellin membrane was then disrupted with a scalpel blade. We collected the yolk in a beaker cooled in ice water. The yolk was subsequently diluted two times (wt/wt) in an isotonic saline solution (0.17 M NaCl). The solution was stirred for 1 hour before centrifugation at 10,000× *g* for 45 minutes at a temperature of 4 °C. The supernatant (plasma) was separated from the sediment and mixed with 40% ammonium sulfate to precipitate the livetins. After 1 hour of stirring in a refrigerator at a temperature of 4 °C, the mixture was centrifuged at 10,000× *g* for 45 minutes to separate the supernatant from sediment. The sediment was discarded and the supernatant dialyzed for approximately 12 hours against distilled water to eliminate any ammonium sulfate. After elimination of ammonium sulfate, the solution was again centrifuged at 10,000× *g* for 45 minutes at 4 °C, and we collected the floating residue that was rich in LDL.

2.4. Extender preparation and cryopreservation

The control extender (Beltsville) was composed of dipotassium phosphate·$3H_2O$ (12.7 g/L), sodium glutamate (8.61 g/L), fructose (5 g/L), sodium acetate·$3H_2O$ (4.3 g/L), n-Tris (hydroxymethyl)methyl-2-aminoethanesulfonic acid (1.9 g/L), potassium citrate (0.64 g/L), monopotassium