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Supplementing rooster sperm with Cholesterol-Loaded-Cyclodextrin improves fertility after cryopreservation

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A B S T R A C T

Little is known about the effects of Cholesterol-Loaded Cyclodextrin (CLC) on post-thaw semen quality in chicken. The aim of the present study is to investigate the efficacy of CLC levels (0, 1, 2 and 3 mg/mL Schramm diluent) on post-thawed semen quality and fertility in two breeds of chicken Pradu Hang Dum (native chicken) and Rhode Island Red. Semen samples of each breed were pooled, divided into 4 aliquots and diluted with Schramm diluents, cooled to 5 °C when DMF was added (6% of final volume). Semen straws were subjected to cryopreservation using the liquid nitrogen vapor method. Post-thawed sperm motility, viability, acrosome integrity, mitochondrial function, and the Malondialdehyde (MDA) level were determined. The fertility of frozen semen was tested by inseminating laying hens. Post-thaw motility between Pradu Hang Dum and Rhode Island Red was no different; but Rhode Island Red had a higher semen viability and live cell intact acrosomes than Pradu Hang Dum ($P < 0.05$). The percentage of high functioning mitochondria in the Pradu Hang Dum was higher than the Rhode Island Red. CLC at 2 and 3 mg/mL supplementation was associated with improved viability of frozen semen; that is, acrosome integrity and mitochondrial function ($P < 0.01$), albeit having no effect on MDA levels. The sperm with 1 mg/mL CLC yielded a significantly better fertility ($P < 0.01$). CLC (1 mg/mL) improved the quality of frozen rooster semen. There was no interaction among breeds and CLC on post-thaw semen quality and fertility.

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1. Introduction

Semen cryopreservation is a crucial biotechnological method of *ex situ* conservation for preventing genetic loss. The application of this technique for cattle has grown rapidly but sperm freezing for poultry is limited because of the high costs of semen processing and subsequent lower fertility [3].

Membrane damage during cooling is a major cause for sperm death during cryo-preservation [21]. Membrane lipid composition plays a significant role in a sperm's response to cooling and warming during cryopreservation [1,6]. Sperm membrane comprises of lipids, cholesterol, and proteins in specific arrangements, and the cholesterol/phospholipid ratio plays a major role in membrane fluidity [8]. Membranes with high cholesterol to phospholipid ratios exhibit lower transition temperatures, which allow the membrane to remain fluid through most of the freezing process and this decreases the amount of membrane damage during cooling and warming [34,23]. Those ratios in roosters are, however, quite low (0.25–0.30) [4,23].

Cyclodextrins are cyclic oligosaccharides formed from starch through cyclodextrin glycosyltransferase action [11]. Cyclodextrins have a hydrophilic surface and a hydrophobic core, which allows the molecules to be soluble in aqueous solutions but allows hydrophobic molecules to solubilize in cyclodextrin. Hydrophobic molecules in cyclodextrin can be solubilized in an aqueous solution, without structural change [7,10]. Pre-loaded Cholesterol (CLC) molecules have been used to incorporate cholesterol in to the sperm membranes and increase the cholesterol content of sperm membranes [20,29]. When the cholesterol content of stallion, boar, ram, and bull sperm is increased by CLC, the cryosurvival rate and quality of the frozen-thawed sperm is increased [2,15,19,20]. This decreases the membrane damage upon warming [5]; therefore, CLC

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treatment prior to cryopreservation may be able to decrease rooster sperm damage after cryopreservation and increase fertility. To our knowledge, there is no published information on the effects of CLC in chicken cryopreserved semen. Our aim therefore was to determine whether treating rooster sperm with CLC prior to cryopreservation would improve post-thaw sperm survival and also to optimize the concentration of CLC suitable in the semen freezing diluent for improving fertility.

2. Materials and methods

2.1. Chemicals

Unless otherwise stated, all chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA). The cell viability test kit was obtained from the Life Technologies (Carl.sbad, CA, USA).

2.2. Animals

Twelve Pradu Hang Dum (Thai Native) and twelve Rhode Island Red, 1 to 2-year-old breeder male roosters were housed in individual cages, fed 130 g/d/b of commercial feed, and given water *ad libitum*. Forty-eight individually housed, commercial laying hens (24 weeks of age) having egg production of 85–95% were fed 110 g/d/b of layer feed and given water *ad libitum*. The animals were reared under natural environment conditions. The study was conducted at the research farm, Department of Animal Science, Faculty of Agriculture, Khon Kaen University; Thailand. The Animal Ethics Committee of Khon Kaen University reviewed and approved the study (Approval No.: 0514.1.12.2/29).

2.3. Preparation of cyclodextrin

Cholesterol-Loaded Cyclodextrin (CLC) was prepared as per Purdy and Graham [27]. Briefly, 1 g of cyclodextrin was dissolved in 2 mL of methanol in a glass tube. In a second glass tube, 200 mg of cholesterol was dissolved in 1 mL of chloroform. The cyclodextrin/methanol solution was added to the cholesterol/chloroform solution and mixed (glass tube 1 into 2). The solution (in a petridish) was then subjected to a stream of nitrogen gas to vapor at the solvent and to obtain a white CLC, which was stored at 5 °C. The CLC working solution was mixed with Schramm diluent, using a vortex mixer for 30 s [27].

2.4. Semen collection

Semen was collected twice a week, by the dorso-abdominal massage method [28]. Semen from an individual rooster was collected in a 1.5 mL micro-tube containing 0.1 mL of Schramm diluent. To maximize semen quality and quantity, the collection was performed by the same person, under the same conditions, at the same time, and using the massage method. Care was taken to avoid contamination of semen with feces, urates, and transparent fluid-all of which lowers the semen quality. Semen samples were selected on the basis of meeting the following criteria: mass motility score ≥4; sperm concentration $\geq 3 \times 10^9$ sperm/mL; sperm viability ≥85%; and, ≥ 90% normal cells in the semen sample [33].

2.5. Semen dilution and cryopreservation

Schramm diluents was used in the current study, comprising of magnesium acetate 0.7 g, sodium glutamate 28.5 g, glucose 5 g, inositol 2.5 g, and potassium acetate 5 g, all of which were dissolved in 1,000mL of double-distilled water (pH = 7.1, Osmotic pressure (O.P.) = 395 mOsm/kg) [31].

Immediately after collection of the semen sample from each breed group, these were pooled separately in 15 mL tubes. Semen in each tube was divided into 4 aliquots and diluted (to 0, 1, 2 and 3 mg/1 mL) of CLC, Schramm-based diluter 1: 3 (v: v) was then cooled to 5 °C for 60 min (1 °C per 3 min). At this temperature, the DMF (N,N-dimethylformamide) was added to a final concentration of 6% (v/v) and then mixed. Semen was immediately loaded into 0.5 mL plastic straws and sealed with Polyvinylpyrrolidone powder (PVP). The number of final sperm concentration in each straw was about 500×10^6. After 15 min of equilibration, the filled straws were laid horizontally on a rack 11 cm above the surface of liquid nitrogen (−35 °C) for 12 min then placed 3 cm above the liquid nitrogen vapor (−135 °C) for 5 min, and subsequently immersed in liquid nitrogen for storage at −196 °C until analyzed. Thawing was achieved at 2-5 °C for 5 min in cool water [33].

2.6. Evaluation sperm quality

The concentration of sperm was determined with a haemocytometer under a light microscope. The computer-assisted sperm analysis system (CASA) (version 10 HIM-IVOS; Hamilton Thorne Biosciences, Beverly, MA USA) has the ability to assess total motility (MOT), progressive motility (PMOT), velocity average path (VAP), velocity straight line (VSL), and velocity curvilinear (VCL) [12]. The percentage viable was determined by SYBR - 14 and Propidiam Iodide (PI) (Live/Dead Sperm Viability Kit, Invitrogen[TH], Thermo Fisher Scientific, Waltham, MA, USA) [24]. The percentage of acrosome integrity and functional mitochondria was determined by PI (Sigma, 81845), 5, 5′, 6, 6’ − tetraethybenzimdazolycarboncyanine iodide (JC- 1; Sigma, C50390) and fluorescein isothiocyanate conjugated peanut agglutinin (FITC-PNA; Sigma, L7381). These were evaluated by flow cytometry (FACSalibur; Becton Dickinson, San Jose, CA, USA) as described by Lange-Consiglio [13] with minor modifications.

2.7. Lipid peroxidation

Malondialdhyde (MDA) concentration as indices of the lipid peroxidation in the semen samples was measured using the Thiobarbituric acid (TBA) reaction. Semen samples from each treatment 250 μL (250×10^6 spz/mL) was centrifuged at 1,800 rpm for 3 min and the supernatant discarded and 1 mL of Schramm diluent added. The semen pellet was added with 0.25 mL Ferrous sulfate (Ajex, 0906251) (0.2 mM) and 0.25 mL ascorbic acid (Sigma, A5960) (1 mM). The mixture was incubated at 37 °C for 60 min after which 1 mL trichloroacetic acid (Sigma, T6399) (15%) and 1 mL thiobarbituric acid (Sigma, T550) (0.375%) were added, and boiled in water for 10 min. Thereafter, the samples were cooled to room temperature to stop the reaction. Finally, the samples were centrifuged to controlled temperature of 4 °C at 5,000 rpm for 10 min and analyzed, using UV–Visible spectrophotometer (Analytikjena Model Specord 250 plus) at 532 nm [24].

2.8. Fertility

The fertility ability of frozen-thawed spermatozoa was tested by inseminating layer hens (48 hens were randomly assigned to 8 groups of 6 hens) once a week with a dose of 0.4 mL. All inseminations were performed between 15.00 and 17.00 Hrs. Eggs were collected during days 2–8 after insemination. Fertility was determined by candling eggs on day 7 of incubation. Six replications of fertility test were carried out.

For the fertile period test, 48 hens (about 80% of hen-day production) were randomly assigned to 3 groups, and inseminated

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