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Original Article

Optimization of Trehalose Concentration in Semen Freezing Extender in Thai Native Chicken Semen

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Abstract

The aim of this study was to find optimal concentration of trehalose supplemented to semen extenders (0, 5, 10, and 15 mM). Ejaculates were collected from 24 Thai native cocks (Pradu Hang Dam; 1 year old), then diluted (1:3) and pooled before allocation to treatments. In the experiment, semen was either cooled to 5ºC after dilution or diluted with a pre-cooled diluents before cooling to 5ºC. Semen freezing was performed in liquid nitrogen vapor at -135ºC and -35ºC, with 6% DMF, and compared for sperm qualities. After thawing, it was measured for total sperm motility and progressive motile by CASA. Sperm parameters in terms of viability (PI), acrosome integrity (FITC-PNA), and mitochondrial function (JC-1) were evaluated using flow cytometry. Malondialdehyde concentration was assessed by TBARS for levels of lipid peroxidation and fertility test was carried out by inseminating layer hens. Results showed that the extender supplemented with 5 mM trehalose produced the greatest sperm motility, progressive motile, intact acrosome membrane, mitochondria activity and fertility rates ($P<0.05$).

Keywords: trehalose, cryopreservation, chicken semen

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Introduction

Artificial insemination is used widely in the ratite industries because there are few realistic alternatives for genetic selection. The ability of sperm to survive cryopreservation and remain functional requires methodologies that are within the biophysical and biological limits defined by the cryo-biological characteristics of each species (Agca and Crister, 2002). In chickens, it is also in terms of the viability and functionality of sperm after cryopreservation, especially the fertility rates from frozen/thawed chicken semen which are consistently low (Long, 2006).

The freezing and thawing of spermatozoa is a complex process that induces several forms of cellular lesions (Purdy, 2006). These lesions have been attributed to cold shock, membrane alteration, osmotic changes and intercellular ice crystals, which may decrease the motility, viability and fertilizing ability of sperm after artificial insemination (Matsuoka et al., 2006). Intracellular ice formation is one of the main damaging factors that reduce the viability of frozen-thawed sperm, and the degree of damage also depends on the composition of the semen extender and nature of the cryoprotectant (Curry et al., 1994). Therefore, cryoprotectants are included in the cryopreservation extender to reduce the damaging effects of the freezing process (Purdy, 2006).

Furthermore, it is known that disaccharides protect the sperm membrane structure from oxidative stress and cold shock damage during the freezing-thawing process. Non-permeant disaccharides have a protective action related to both osmotic effect and specific interactions with membrane phospholipids (Liu et al., 1998).

Trehalose (α-D-glucopyranosyl-α-D-glucopyranoside) is found in a number of plants and animals that can resist dehydration or freezing (Woelders et al., 1997). The action of trehalose appears to be connected with its ability to replace water at the membrane/solution interface (Bakas and Disalvo, 1991). During the dehydration and rehydration of cryopreservation, trehalose interacts with the plasma membrane phospholipids, increases its fluidity, reorganizes the sperm membrane and induces depression in the membrane phase transition temperature of dry lipids. Thus, their sugars probably play a key role in preventing deleterious alteration to membranes during reducing-water state (Fernandes-Santos, 2007). Trehalose is not an antioxidant and has been shown to have a protective effect on the deleterious effects of low temperature on the membranes of bulls (Chen et al., 1993; Woelders et al., 1997; Uysal et al., 2007; Sariozkan et al., 2009; Hu et al., 2010), mice (Storey et al., 1998), rams (Lopez-Saaz et al., 2000; Aisen et al., 2002; Bucak amd Tekin, 2007; Bucak et al., 2007; 2008; Uysal and Bucak, 2009; Nur et al., 2010; Cirit et al., 2013; Najafi et al., 2013), buffalo (Reddy et al., 2010), boars (Funahashi and Sano, 2005; Gutierrez-Perez et al., 2009; Hu et al., 2009), dogs (Yildiz et al., 2000; Yamashiro et al., 2007; Michael et al., 2007; Martins-Bessa et al., 2009), rabbits (Dalimata and Graham, 1997; Kozdrowski, 2009) and goats (Aboagla and Terada, 2003; Atessshin et al., 2008; Khalili et al., 2009; Tuncer et al., 2013). It has been of interest to compare trehalose as a membrane stabilizer with antioxidants to ascertain which action, anti-peroxidative or membrane stabilization, may provide the greater protective effect on the sperm (Bacak and Tekin, 2007). Until now, few reports have evaluated the effects of trehalose supplementation in semen extender on chicken spermatozoa after the thawing of sperm. The aim of the present systematic study was to investigate the effects of the addition of trehalose at different doses on the maintenance of sperm motility, intact acrosome membrane, mitochondria activity, malondialdehyde concentration and fertility on post-thaw cryopreserved Thai native chicken spermatozoa.

Materials and Methods

Animal: Twenty-four mature (one year old) Thai native cocks (*Gallus domesticus*; Pradu hang dam) were kept in individual cages. The cockerels were fed 130 g/head/day, and water was provided *ad libitum*. The animals were reared under natural environmental conditions in which they received a natural light dark photo period (11.14L: 12.46D to 13.01L: 11.59D) throughout the experiment.

Semen collection: Semen from 24 individual Thai native cocks was collected two times a week, by the dorso-abdominal massage method (Burrows and Quinn, 1937). Semen from an individual cock was collected in a 1.5 mL micro tube containing 0.1 mL Schramm diluents composed of 0.7 g magnesium acetate, 28.5 g sodium glutamate, 5 g glucose, 2.5 g inosital and 5 g potassium acetate dissolved in 1,000 mL double-distilled water (Schramm, 1991). To maximize semen quality and quantity, the collection was always performed by the same people, under the same conditions, time, and massage method. Ejaculates having good motility (≥ 85%) were used in this study. This research project was approved by Animal Ethics Committee of Khon Kaen University (Approval No: 0514.1.12.2/31).

Semen processing: Schramm diluent was used as the extender. Each pooled ejaculate was split into 4 equal aliquots, diluted with Schramm extender supplemented with trehalose (Sigma-Chemical Co., St. Louis, USA, T0167) (0, 5, 10 and 15 mM), cooled to 5°C in 60 min, and then diluted with DMF (N, N-Dimethylformamide from Sigma-Chemical Co., St. Louis, USA, D-4551) to a final concentration of 6% (v/v) in diluted semen. After 15 min of equilibration, 500 µl aliquots of treated semen were immediately loaded into 0.5 mL plastic straws to constitute a sperm concentration of 500×10^6/straw (IMV ref. 005569), and sealed with polyvinylpyrrolidone (PVP) powder (IMV ref. 018818). After 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 liquid nitrogen vapor (-135°C) for 5 min, and subsequently immersed in liquid nitrogen (Vongpralub et al., 2011). After storage, the straws were thawed individually in an ice water bath at 5°C for 5 min and then evaluated for sperm qualities.

Evaluation of post-thaw sperm motion parameters: Post-thaw sperm motion parameters were determined

using computer assisted sperm analysis (CASA) (HTM-IVOS Model 10 Spermatozoa Analyzer; Hamilton Thorne Biosciences, Beverly, MA, USA) attached with Olympus software to process video material recorded in "avi" format. For each sample, two slides (maintained at 37°C) were filled with 5 µL diluted semen and three fields per slide were recorded for 10 sec using a 10x phase-contrast objective (Olympus) in conjunction with a digital camera (Olympus DP 71/25). The instrument setting for computer-assisted semen analysis (CASA) was as follows: apply sort = 0, frames acquired = 30, frame rate = 60 Hz, minimum contrast = 25, minimum cell size 4 pixels, minimum static contrast = 15, straightness (STR) threshold = 80.0%, average path velocity (VAP) cut-off = 5 µm/sec, Prog. Min VAP = 20 µm/sec, VSL cut-off = 20 µm/sec, cell size = 4 pixels, cell intensity = 50, static head size = 0.72 to 8.82, static head intensity = 0.14 to 1.84, static elongation = 0 to 47, slow cell motile = Yes, magnification = 1.92, video frequency = 60 frames/sec, bright filed = No (i.e. no bright field), chamber depth = 20 µm, field selection mode = Auto, and integration time = 1 frame. The following motility characteristics were determined: percentage of motile sperm (PMOT), curvilinear velocity (VCL; in µm/sec, average velocity measured over the actual path), VAP (in µm/sec, velocity/average position of sperm), progressive velocity (VSL; in µm/sec, straight-line distance between the beginning and the end of the track/time elapsed), STR coefficient ([VSL / VAP] x 100), and linearity (LIN; departure of the cell track from straight line [VSL / VCL x 100]).

Evaluation of acrosome integrity and mitochondrial function by Fluorescent multiple staining (FMS): Propidium Iodide (PI; Live/dead® sperm viability kit L7011 Invitrogen USA), a red-fluorescent nuclear and chromosome counterstain, was used to identify dead cells. To make a stock solution the solid form was dissolved in deionized water to a concentration of 1 mg/mL (1.5 mM).

Fluorescein isothiocyanate-conjugated peanut agglutin (FITC-PNA; Sigma L7381) was used to determine acrosome status of viable spermatozoa. FITC-PNA intensely labeled the acrosome region of acrosome-reacted spermatozoa. A stock solution was prepared by dissolving 2 mg of FITC-PNA in 1 mL PBS pH 7.4.

5, 5′, 6, 6′-tetrachloro-1, 1′, 3, 3′ tetraethylbenzimidazolyl-carbocyanine iodide (JC-1; Sigma, C50390) is a selective mitochondrial stain. Cells with a high membrane potential form J-aggregates, thus showing high red fluorescence with JC-1. Cells with a low membrane potential JC-1 maintained their monomeric form, thus showing only green fluorescence. A stock solution of 0.153 mM in dimethylsulfoxide (DMSO) was prepared.

The protocol of multiple fluorescent staining was as follows: semen samples of semen were diluted to a concentration $15x10^6$ spermatozoa/mL with Schramm diluents. Then, 3 µl of PI, 5 µl of FITC-PNA and 2 µl of JC-1 were added to 300 µl aliquots of the diluted semen. The samples were incubated at room temperature for 8 min in the dark. Then, they were centrifuged at 1,200 x g for 3 min and sperm pellets were resuspended in 500 µl of Schramm extender before cytometric analysis. Measurement was performed on a FACSCalibur (Becton Dickinson, San Jose, CA, USA) flowcytometer in a triple filter, showing set: UV-2E/C (excitation 340-380 nm and emission 435-485 nm), B-2E/C (excitation 465-495 nm and emission 515-555 nm) and G-2E/C (excitation 540-525 nm and emission 605-655 nm) (Adapted from Andrade et al., 2007; Partyka et al., 2011; Consiglio et al., 2013).

Production of reactive oxygen species: Sperm oxidative levels were determined using the thiobarbituric acid (TBA) reaction $250x10^6$spz/mL. Each treatment was incubated in a solution containing 2.78% ferrous sulfate7H_2O (Ajex, 0906251), 0.1 mL 0.22% butylatedhydroxytoluene (Sigma, B1378) at 37°C for 60 min. The reaction was stopped by adding 1 mL of 35% trichloroacetic acid (Sigma, T6399) and kept on ice for 15 min. The samples were centrifuged at 7,800 x g for 15 min and the supernatant was retained. The progress of endogenous peroxidation was followed by adding 1 mL of 0.36% thiobarbituic acid (Sigma-T550-0) to 2 mL of supernatant. The mixture was boiled for 10 min and allowed to cool. Then, the production of reactive oxygen species was measured by a Carry Conc. UV-Visible Spectrophotometer (Specord 250 plus, Analytikjena) and absorbance levels were acquired by spectrophotometry at 532 nm (Partyka et al., 2007).

Fertilizing ability test: The fertilizing ability of frozen-thawed spermatozoa was tested by inseminating layer hens aged 43 weeks old once a week with a dose of 0.4 mL. All insemination was performed at 15.00-17.00. 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.

Statistical analysis: The experiment was conducted as a randomized complete block design (RCBD) and differences in number of particular categories of spermatozoa in frozen-thawed semen were analyzed with ANOVA and Duncan's multiple range tests. Results are presented as mean±SEM of measurements on samples from 6 replicate determinations ($P<0.05$).

Results

Sperm characteristics: The effects of varying trehalose concentrations on frozen-thawed chicken sperm motility, progressive motile, intact acrosome membrane and mitochondria activity are shown in Table 1, Fig 1a and 1b. The 5 mM trehalose treatment resulted in greater sperm motility, progressive motility, intact acrosome membrane and mitochondria activity.

Production of lipid peroxidation: The effect of trehalose on lipid peroxidation on thawed chicken semen is shown in Table 2. The addition of trehalose did not cause significant difference in the level of malondialdehyde compared to the control group with no addition.

Fertility ability: This study showed that the effect of trehalose concentrations on the fertility ability of sperm frozen with 5 mM trehalose was significantly higher than that of the control, 10 mM and 15 mM groups (P<0.05) as shown in Table 2.

Table 1 Motility parameters, acrosome integrity and functional mitochondria of frozen-thawed chicken spermatozoa (results expressed as mean ± SE; n=6).

Parameters (%)	Trehalose concentration (mM/mL)			
	0	5	10	15
Total motility	47.00±1.12^{b}	53.00±1.48^{a}	36.75±1.70^{c}	35.25±1.50^{cd}
Progressive motile	27.25±1.06^{a}	27.85±1.00^{a}	26.50±1.73^{b}	23.25±1.70^{c}
Live with intact acrosome	28.50±0.70^{b}	33.30±1.27^{a}	24.70±0.84^{c}	24.50±0.84^{c}
Live with ruptured acrosome	19.70±0.14^{a}	17.20±1.27^{ab}	13.75±0.77^{c}	16.20±1.55^{b}
Live with active mitochondria	45.40±1.55^{ab}	48.75±0.35^{a}	39.20±0.28^{b}	36.80±0.42^{c}
Live with inactive mitochondria	2.80±0.42	1.80±0.42	1.65±0.35	1.50±0.42

Different letters ($^{a,\ b}$) within rows indicate significant differences (P<0.05).

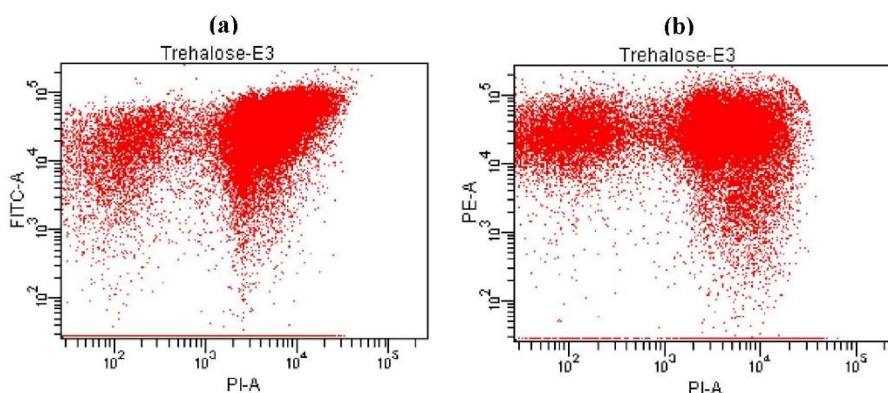

Fig 1 Flow cytometric detection of frozen-thawed chicken spermatozoa **(a)** stained with fluorescein isothiocyanate conjugated peanut agglutinin (FITC-PNA) and propidium iodide (PI) and **(b)** chicken spermatozoa stained with 5, 5', 6, 6'- tetrachloro 1, 1', 3, 3' tetraethylbenzimidazolyl-carbocyanine iodide (JC-1) (100,000 spz).

Table 2 Malondialdehyde concentration as level of lipid peroxidation (MDA) and percentage of fertility test in frozen-thawed chicken semen (mean ± SE; n=6).

		MDA (µM/mL/50x10^{6}spz)	Number of set eggs	Fertility (%)
Trehalose (mM/mL)	0	1.42±0.99	235	77.78±1.79^{b}
	5	1.48±0.26	243	87.67±1.26^{a}
	10	1.62±0.90	234	72.29±1.58^{c}
	15	1.54±0.20	236	70.75±1.47^{c}

Different letters ($^{a,\ b}$) within columns indicate significant differences (P<0.05).

Discussion

When cells are frozen, they are subjected to various stresses such as cold shock and oxidative stress, which arise through ice crystallization due to membrane changes (Bilodeu et al., 2000). The efficacy of avian semen cryopreservation depends on many factors, mainly those associated with species, breed, freezing medium (extender and cryoprotectants), procedures of semen equilibration, and freezing and thawing rates and temperatures (Lukaszewicz, 2006). Trehalose can improve the antioxidant action in semen extender, resulting in better protection of sperm plasma membrane in semen cryopreservation (Aisen et al., 2005). The cryoprotective capacity of trehalose varies depending on the concentration of supplementation in the extenders (Naing et al., 2010).

In this study, it was clear that the supplementation of trehalose to the extender improved chicken semen quality variables and fertilizing ability.

The extender supplemented with 5 mM trehalose resulted in the greatest sperm motility, progressive motile, intact acrosome membrane, mitochondria activity and fertility rates. The current findings were in agreement with many studies and indicated the favorable effects of trehalose on post-thaw sperm quality.

Trehalose has a protective action related to the osmotic effect and to specific interactions with membrane phospholipids, which causes media hypertonic, thereby minimizing the degree of sperm cell injury during the freeze-thaw process (Storey et al., 1998). Sperm plasma membrane is one of the primary sites of damage induced by cryopreservation. It is assumed that trehalose enables the plasma membrane to be less vulnerable to cryo-damage during the freezing and thawing process. Most previous studies in boar (Hu et al., 2009), bull (Hu et al., 2010), goat (Khalili et al., 2009) and ram (Jafaroghli et al., 2011) revealed that trehalose supplementation in semen extender enhanced membrane integrity. The presence of trehalose in an extender is likely to modulate membrane fluidity by inserting itself into membrane phospholipids bilayer, thus it renders membrane more stable during freezing (Aboagla and Terada, 2003). Aisen et al. (2002) observed a favorable effect of the reduced trehalose concentration on sperm motility and a deleterious effect of the greater trehalose concentration, which are consistent with our previous observations carried out in boar semen (Hu et al., 2009).

The integrity of sperm acrosome membrane associated with sperm motility can be expected to be destroyed by high concentration of trehalose. In the present study, the greatest protective effects of trehalose were at the concentration of 100 mM, and a much reduced extent at 200 mM. The latter concentration resulted in increased osmolarity of the extender as the extender was deleterious to the sperm cells (Hu et al., 2009). When trehalose concentration was 200 mM, the percentages of motile sperm and intact acrosomal membrane sperm of frozen-thawed bovine semen decreased. Reddy et al. (2010) reported that trehalose had the capacity to reduce this cryocapacitation and maintained the acrosomal integrity. The results of this study showed that 100 mM trehalose supplementation to the extender could significantly reduce ($P<0.05$) what? after freezing and thawing. Similarly, studies in dogs (Yildiz et al., 2000) and boar (Hu et al., 2009) concluded that trehalose helped to reduce acrosomal abnormalities occurring during cryopreservation. Because trehalose helps to diminish acrosomal damage and enhance membrane fluidity during cryopreservation, it ultimately affects fertility. A limited number of studies have been conducted to evaluate the fertility of sperm frozen with trehalose.

In this study, the extender supplemented with 5 mM trehalose resulted in adjective? fertilizing ability (87.67%) in the frozen/thawed chicken semen. Sztein et al. (2001) reported that the frozen/thawed sperm cryoprotected with trehalose retained significantly better *in vitro* fertility (79%) than the control (11%) in mice; however, percentage of offspring born was the same. Similarly, 45 to 47% lambing was obtained in ewes inseminated with sperms cryopreserved in 100 mM trehalose extender, which was 2.5 times higher than the control (Aisen et al., 2002). In another study, a significant difference was recorded in fertility rates (49.8% vs 16.7%) in ewes following AI with frozen-thawed 100 mM trehalose treated or control semen (Jafaroghli et al., 2011). In contrast, the addition of trehalose to an extender did not improve the fertility of frozen-thawed bull semen (Foote et al., 1993) and European brown hare semen (Kozdrowski, 2009).

This report examined the effect of trehalose on the cryosurvival of chicken semen. The addition 5 mM dose of trehalose will be useful for increasing the post-thaw quality and fertility rate of Thai native chicken semen.

In conclusion, the presence of balanced amounts of trehalose (5 mM) in the extender during the freezing-thawing process could provide chicken semen with quality and fertility.

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บทคัดย่อ

ผลของการเสริมทรีฮาโลสระดับที่แตกต่างกันในน้ำยาเจือจางน้ำเชื้อไก่ที่ผ่านการเก็บรักษาด้วยวิธีการแช่แข็ง

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การศึกษานี้มีวัตถุประสงค์เพื่อศึกษาผลของการเสริมทรีฮาโลสที่ระดับ 0, 5, 10 และ 15 มิลลิโมลในน้ำยาเจือจางสูตร Schramm ในไก่พันธุ์ประดู่หางดำอายุเฉลี่ย 1 ปี นำตัวอย่างน้ำเชื้อมาเจือจางในน้ำยาเจือจางที่อัตราส่วน 1:3 และใช้ DMF เป็นสารป้องกันความเสียหายจากการแช่แข็งที่ระดับ 6 เปอร์เซ็นต์ของปริมาตรน้ำเชื้อ ทำการลดอุณหภูมิไปที่ 5 องศาเซลเซียส และบรรจุน้ำเชื้อในหลอดพลาสติกขนาด 0.5 มิลลิลิตร ซึ่งมีอสุจิประมาณ 500×10^6 ตัว/หลอด ทำการแช่แข็งโดยวิธีอังไอไนโตรเจนเหลวที่อุณหภูมิ -35 องศาเซลเซียส และ -135 องศาเซลเซียส จากนั้นเก็บรักษาในไนโตรเจนเหลว ภายหลังการละลายน้ำเชื้อทำการประเมินอัตราการเคลื่อนที่ของอสุจิโดย CASA ประเมินอัตราการรอดชีวิต ความสมบูรณ์ของอะโครโซม และการทำงานของไมโตคอนเดรีย โดยการย้อมสีฟลูออเรสเซนต์ชนิด PI FITC-PNA และ JC-1 จากนั้นนำไปวิเคราะห์ด้วย flow cytometer ประเมินอัตราการเกิด ROS โดยวิเคราะห์จากความเข้มข้นของปริมาณ Malondialdehyde โดยวิธี TBARS และทดสอบความสมบูรณ์พันธุ์และอัตราการผสมติดโดยการผสมเทียมในแม่ไก่ไข่พันธุ์ทางการค้า จากการศึกษาพบว่าการเสริมทรีฮาโลสที่ระดับ 5 มิลลิโมลลงในน้ำยาเจือจางสามารถปรับปรุงอัตราการเคลื่อนที่ อัตราการรอดชีวิต ความสมบูรณ์ของอะโครโซม การทำงานของไมโตคอนเดรียภายหลังการเก็บรักษาด้วยวิธีแช่แข็ง และอัตราการผสมติดได้ ($P<0.05$)

คำสำคัญ: ทรีฮาโลส การเก็บรักษาด้วยวิธีการแช่แข็ง น้ำเชื้อไก่

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