Sucrose increases the quality and fertilizing ability of cryopreserved chicken sperms in contrast to raffinose

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ABSTRACT Chicken semen conservation is an important tool for programs of genetic diversity management and of endangered breeds' conservation. However, the method still needs to be improved in order to be applied in a wide variety of environments and breeds. Our objective was to compare the effects of 2 external cryoprotectants saccharides (sucrose and raffinose) on the sperm freezability of a Thai local breed, Pradu Hang Dum, in which semen was frozen with a simple freezing method using nitrogen vapors and dimethyl formamide (DMF). Thirty-six males were selected on their motility vigor score for the experiments. In a first experiment, a large range of sucrose and raffinose doses were tested. Semen quality was evaluated after incubation at 5°C or after cryopreservation in straws in the saline Blumberger Hahnen Sperma Verdünner diluent + DMF (6% v/v) with or without sucrose/raffinose. The best targeted doses of sucrose and raffinose were then kept for experiment 2 that was focused on cryopreserved semen. In this experiment, semen quality was measured on frozen-thawed sperm: different objective motility data evaluated by computer-assisted sperm analysis (CASA), membrane integrity, acrosome integrity, mitochondria function evaluated using flow cytometry, lipid peroxide production assessed by the thiobarbituric acid test. Fertility obtained with frozen-thawed semen supplemented or not with sucrose or raffinose was also evaluated after artificial insemination of laying hens.

The presence of sucrose at the osmotically inactive dose 1 mmol significantly increased the vigor motility, membrane integrity, acrosome integrity, and mitochondrial functions of frozen-thawed sperm ($P < 0.05$), and showed the highest levels of fertility after sperm cryopreservation (91% vs. control 86%, $P < 0.001$). Raffinose showed negative effects on in vitro semen quality from 1 to 100 mmol. Fertility was also negatively ($P < 0.001$) affected by raffinose (fertility rate 66 to 70%).

We thus showed in the present study the high success of a simple chicken sperm cryopreservation method with an external cryoprotectant easily available and cheap, the sucrose, used at an osmotically inactive low concentration.

Key words: sucrose, semen cryopreservation, chicken, fertility

2019 Poultry Science 98:4161–4171
http://dx.doi.org/10.3382/ps/pez196

INTRODUCTION

Genetic resources have to be optimized in order to reach a better domestic animal genetic diversity and to preserve endangered animal genomes (Blesbois et al., 2008). This requires conservation programs to take animal management into consideration. In poultry, in addition to in vivo management, in vitro conservation is strategic in order to secure genetic diversity of a wide range of lines and breeds, and/or to contribute to create new resources (Blesbois et al., 2007, 2008; Varadi et al., 2013; Madeddu et al., 2016). The more feasible and available method for management of reproductive cells in bird populations is, therefore, semen cryopreservation (Blesbois et al., 2007). Other methods including primordial germ cells and gonadic tissues technologies are still in progress but may be seen as methods that are complementary to semen cryopreservation (Liptoi et al., 2013; Nakamura, 2016; Sztan et al., 2017; Thélie et al., 2018). Different freezing procedures have been

Received November 26, 2018.
Accepted March 21, 2019.

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developed to cryopreserve avian semen (Blesbois et al., 2007; Santiago-Moreno et al., 2012; Rakha et al., 2016, 2017a; 2018a,b,c; Thananurak et al., 2016; Chuaychunoo et al., 2017; Thananurak et al., 2017). However, simplification of the more efficient common methods of chicken sperm cryopreservation is still needed in order to make them available to large environmental and breeding conditions. In previous research assessing different freezing protocols, a programmable freezing machine was used which is not always available in field conditions (Santiago-Moreno et al., 2011; Blanco et al., 2012; Thélie et al., 2018).

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, extreme osmotic changes, intracellular ice crystals, reactive oxygen species, which decrease the membranes permeability and integrity, motility, viability, and fertilizing ability of sperm after artificial insemination (**AI**) (Matsuoka et al., 2006). Intracellular ice formation is one main damage factor that reduces the viability of frozen-thawed sperm, and the degree of damage also depends on the composition of the extender and nature of the cryoprotectant (Curry et al., 1994). Therefore, cryoprotectants are included in the cryopreservation extenders in order to reduce the damaging effects of the freeze/thaw process (Purdy, 2006). Bird sperm show specific characteristics that need added adaptations for sperm cryopreservation. They contain the same intra-cellular organelles as in all amniotes, including mammals. However, in contrast to most mammals, they are filiform cells with a very low cytoplasmic content and a long flagellum adapted to their long trip in the female tract before reaching the site of fertilization (Etches, 1996; Blesbois and Brillard, 2007; Bakst, 2011). They consequently contain a relatively low amount of intracytoplasmic water and very high proportions of cellular membranes that make them highly sensitive to the membrane injuries that may occur during the changes of osmolarity needed for the freeze-thaw process (Long, 2006; Blesbois, 2012). Thus, avian sperm are especially intolerant to volume and osmotic changes compared to mammalian sperm.

Many sugars may play a role in the protection of sperm during the freeze-thaw process. Among them, di- and tri-saccharides such as sucrose, trehalose (disaccharides), and raffinose (trisaccharide) are considered as external cryoprotectants that do not enter the cell by contrast to internal cryoprotectants such as glycerol, dimethyl sulfoxide, dimethyl formamide (**DMF**), dimethyl acetamide, and ethylene glycol (**EG**) and also some monosaccharides. The role of di- or tri-saccharides as external cryoprotectants is complex. At high concentration, they are involved in the dehydration of the cell needed to avoid intracellular ice crystals formation (Abdelhakeam et al., 1991; Olsson et al., 2016). They have been also suggested to be involved in the van der Waals effect, and to "coat" parts of the plasmic membrane, allowing a higher protection of the membrane structure (Sousa, 1995; Taylor and Zografi, 1998; Lerbret et al., 2005). Their protective effects depend on many factors, i.e., the saccharide molecular weight, storage temperature, and the composition of the extender (Garde et al., 2008). Different disaccharides (sucrose/trehalose) and/or trisaccharides (raffinose) have been successfully incorporated into ram, red deer, and bull sperm cryopreservation extenders (El-Sheshtawy et al., 2015; Fernandez-Santos et al., 2007; Bucak et al., 2010; Tuncer et al., 2010, 2011; Bucak et al., 2013). In avian species, disaccharides (sucrose) showed contrasted effects on the success of sperm cryopreservation (Blanco et al., 2011; Mosca et al., 2016; Miranda et al., 2017; Brown et al., 2018) while nothing is known about the effect of raffinose on sperm cryopreservation.

In the present study, our aim was to examine the effects of a disaccharide (sucrose) and a trisaccharide (raffinose), used at inactive or active osmotic levels, on the success of chicken sperm in vitro conservation. A simple sperm cryopreservation method using nitrogen vapors and DMF internal cryoprotectant was used. We successively examined the effect of a wide range of sucrose and raffinose doses on unfrozen and frozen-thawed semen in vitro quality (motility vigor score and membrane integrity/viability). Then we restrained the observations to the most promising dose that we tested on frozen-thawed semen in vitro (objective motility measurements with CASA, membrane integrity/viability, acrosome integrity, mitochondria function, lipid peroxide production), and in vivo (fertility rate) quality.

MATERIALS AND METHODS

All experimental procedures used were approved by the Animal Ethics Committee of Khon Kaen University, based on the Ethic of Animal Experimentation of National Research Council of Thailand (Approval No: 0514.1.75/22).

Chemicals

Unless otherwise indicated, all chemical used in this study were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Animals

A flock of 60 adult males of the Thai native breed (Pradu Hang Dum) was bred at the Khon Kaen University facilities. Thirty-six of these males were chosen for our experiments on their semen capacities (vigor motility, semen production). Animals were kept individually in cages. The birds were offered commercially available poultry cock breeder food (130 g/364 Kcal/day) and were exposed to natural light (12L:12D). Fresh water was available ad libitum

throughout the experimental period. They were 25 wk old at the start of the experiment.

The females were 60 hens of a commercial layer type (Isa Brown; Betagro Public Company Limited, Thailand). They were 25 wk old at the beginning of the experiment. They were exposed to natural photoperiod (12L:12D) and received a laying hen diet supplemented with calcium of 110 g/308 Kcal/day. For all the animals, care was taken to ensure maximized welfare (constant care, gentle manipulations)

The study was conducted at the research farm of the Department of Animal Science, Faculty of Agriculture, Khon Kaen University, Thailand.

Semen Collection and Preparation

Semen was routinely collected twice a week, by the dorso-abdominal massage method (Burrow and Quinn, 1937). This technique respects welfare and is not invasive: the animal are simply caught by hand and free to go after the abdominal massage, without suffering any injury. By establishing a good relationship between the human staff of Khon Kaen University and the animals, the animals enjoyed this relationship and the males gave semen very easily and without any stress. Semen from individual cocks was collected in a 1.5 ml microtube. Semen samples were evaluated under a microscope within 15 min following collection, and were selected on the basis of the following criteria: mass motility score $\geq$ 4 (score range 0 to 5, phase contrast microscope $\times$ 40); sperm concentration $\geq 3 \times 10^9$ sperm/mL (hemocytometer counting method); and membrane integrity/viability (SYBR-14 and propidium iodide [**PI**]; Live/dead sperm viability kit L7011, Invitrogen, USA) $\geq$ 90%. To maximize semen quality and quantity, the collection was always performed by the same people, under the same conditions, time, and massage method. Special care was taken to avoid contamination of semen with feces, urates, and transparent fluid, which decrease semen quality.

Extender Preparation and Processing

Experiment 1: Effects of a Large Range of Sucrose and Raffinose Doses Two sub-experiments, one for 0, 0.25, 0.5, and 0.75 mmol (each sucrose and raffinose) and another for 1, 5, 10, 15, 20, 25, 50, 75, and 100 mmol (each sucrose and raffinose), were made. Semen samples were pooled and then separated in aliquots for sub-experiment 1 or aliquots for sub-experiment 2, each aliquot was assigned to 1 treatment. Semen aliquots were diluted 1:2 with Blumberger Hahnen Sperma Verdünner (**BHSV**)-based diluent (5 g glucose, 2.5 g inositol, 28.5 g sodium glutamate, 0.7 g magnesium acetate tetrahydrate, 5 g potassium acetate, all of which were dissolved in 1,000 mL of double-distilled water, Schram, 1991) supplemented with different levels of di- or/and trisaccharide.

Table 1. pH and osmolality of the BHSV diluent supplemented with different doses of sucrose and raffinose.

Factor		pH	Osmotic pressure (mOsm/kg)
Control	0	6.8	422
	0.25	6.8	424
	0.5	6.8	426
	0.75	6.8	426
	1	6.8	426
	5	6.8	431
Sucrose (mmol)	10	6.8	436
	15	6.8	440
	20	6.8	447
	25	6.8	451
	50	6.8	476
	75	6.8	505
	100	6.8	534
	0.25	6.8	424
	0.5	6.8	424
	0.75	6.8	425
Raffinose (mmol)	1	6.8	425
	5	6.8	430
	10	6.8	436
	15	6.8	442
	20	6.8	446
	25	6.8	452
	50	6.8	479
	75	6.8	503
	100	6.4	528

The diluent osmolality was assessed by an osmometer (FISKE Mark 3 Osmometer; FISKE Associates, Norwood, Massachusetts, USA). The pH was assessed by pH meter (CRITON Instrument, S.A., Spain). Only the doses of sucrose and raffinose higher than 10 mmol showed a consistent increase in the osmolarity of the diluent (Table 1). The pH of the diluent was not affected by any concentration of the 2 sugars tested (Table 1).

The pooled diluted semen samples were separated in 2 groups of samples; one group for unfrozen semen was cooled down from 25 to 5°C in 1 h (1°C per 3 min) and equilibrated 24 h at 5°C with or without different sacharride doses. Another group for frozen semen was diluted in BHSV with or without different saccharide doses (Sigma Aldrich, St.Louis, MO, USA). DMF was added to each semen-extender treatment (final concentration of 6% DMF) in the diluted semen samples (final dilution 1:3) cooled down from 25 to 5°C for 1 h. Semen was then immediately loaded into 0.5 mL plastic straws (IMV Technologies, L'Aigle, France), sealed with polyvinylpyrrolidone (**PVP**) powder (IMV Technologies, L'Aigle, France) and equilibrated at 5°C for 15 min (mean sperm concentration per straw: 1×10^3 million sperm). After equilibration, the filled straws were laid horizontally on a rack 11 cm above the surface of LN_2 (–35°C) for 12 min, then placed 3 cm above liquid nitrogen vapor (–135°C) for 5 min, and subsequently immersed in LN_2 as previously described (Vongpralub et al., 2011). Semen straws were transferred to a LN_2 container for storage (mean of 4 D). Sperm was thawed for 5 min in a water bath adjusted to 5°C. After thawing, the straws were quickly opened and semen

transferred to a 1.5-mL microtube and then evaluated for vigor score and membrane integrity. Sperm quality were determined in 6 replicate in both incubated unfrozen and frozen-thawed semen samples.

Experiment 2: Effects of Targeted Doses of Sucrose and Raffinose Semen samples were pooled, and split into 7 aliquots, each one assigned to 1 treatment. Semen aliquots were diluted with BHSV-based extender and supplemented with different levels of sucrose and raffinose (0, 1, 5, and 10 mmol). After dilution and cooling from 25 to 5°C for 1 h, samples were frozen as stated above for experiment 1. Three days later, frozen semen samples were thawed. Sperm quality and fertility were determined in 9 replications.

Analysis of Vigor Motility Score

Vigor motility scores (1 to 5) were assessed using an arbitrary scale of 0 to 5 (0—no movement, 1—little movement, 2—no swirls, but prominent individual movement, 3—slower swirls and eddied, 4—medium swirls and eddied, and 5—very strong movement and rapid dark swirls). A drop of 15 μL of semen sample (semen diluted with BHSV extender; 1:3) was dropped on slide and observed under microscope at 10× magnification.

Analysis of Membrane Integrity/viability

Sperm membrane integrity was assessed with dual fluorescent probes, SYBR-14 and PI (LIVE/DEAD Sperm Viability Kit; Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) according to the method adapted from Chalah et al. (1999). Briefly, each sample was diluted to a concentration of 150 million sperm/mL. Portions of the diluted samples were dropped into a cytometric tube and 5 μL of SYBR-14 in distilled water at a ratio of 1:49. Samples were mixed and incubated at room temperature for 10 min. The cells were counterstained with 5 μL PI for 5 min and then fixed with 30 mL 20% formaldehyde. The sperm was then evaluated under a fluorescent microscope IX71 (Olympus, Tokyo, Japan) in experiment 1, or by flow cytometry in experiment 2 (FACSalibur; Becton Dickinson, San Jose, CA, USA). The PI—negative and SYBR-14—positive population showing green fluorescence was considered to be live, with intact sperm plasma membrane.

Analysis of the Post-thaw Sperm Motion Parameters

Analysis of post-thaw sperm motion parameters was determined using CASA (HTM-IVOS Model 10 Spermatozoa Analyzer; Hamilton Thorne Biosciences, Beverly, MA, USA). The frozen sperm were thawed in water bath at 5°C for 5 min. For each treatment, the frozen-thawed samples were diluted again in the same extender that used for given each treatment (i.e., BHSV for the control treatment; BHSV supplemented with a given saccharide dose for each other treatment) at a ratio of 1:15. For each sample, 2 slides (maintained at 25°C) were filled with 5 μL diluted semen and 3 fields per slide were recorded for 10 s. The instruments setting for CASA were 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, threshold = 80.0%, average path velocity cutoff = 5 μm/s, Prog. Min VAP = 20 μm/s, VSL cutoff = 20 μm/s, 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 total motile sperm (**MOT**), progressive motile sperm, velocity/average position of sperm (**VAP**; in μm/s), progressive velocity (**VSL**; in μm/s, straight-line distance between the beginning and the end of the track/time elapsed).

Analysis of Acrosome Integrity and High Mitochondrial Function of Viable Sperm

This analysis was made with 3 fluorescence staining: fluorescein isothiocyanate-conjugated peanut agglutin (**FITC-PNA**; Sigma L7381) was used to determine the acrosome status of viable sperm (2 mg/mL); 5, 5′, 6, 6′-tetrachloro-1, 1′, 3, 3′ tetraethylbenzimidazolylcarbocyanine iodide (**JC-1**; Sigma, C50390) is a selective mitochondrial stain (1 mg/mL) and PI (Live/dead sperm viability kit L7011 Invitrogen, USA) was used to identify dead cells in a population (1 mg/mL).

The protocol of multiple fluorescent staining was as follows: samples of semen were diluted to a concentration 150 million of sperm/mL with BHSV-based extenders. Then, 300 μL aliquot from diluted semen was added 3 μL of PI, 5 μL of FITC-PNA and 2 μL of JC-1. Then, samples were incubated at room temperature for 8 min in the dark. Samples were then centrifuged at 1,200× g for 3 min and the sperm pellets were resuspended in 500 μL of BHSV-based diluent before cytometric analysis. Measurements were performed on a FACSCalibur (Becton Dickinson, San Jose, CA, USA) flow cytometer in a triple filter, showing set: UV-2E/C (excitation 340 to 380 nm and emission 435 to 485 nm), B-2E/C (excitation 465 to 495 nm and emission 515 to 555 nm), and G-2E/C (excitation 540 to 525 nm and emission 605 to 655 nm) (Adapted from Andrade et al., 2007; Partyka et al., 2011; Consiglio et al., 2013).

Production of Lipid Peroxide

Sperm lipid peroxides were determined using the thiobarbituric acid reactive test (**TBARS**). Semen aliquots of each treatment were incubated in the

presence of 2.78% ferrous sulfate/7H_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 35% trichloroacetic acid (Sigma, T6399) and kept on ice for 15 min. Samples were centrifuged at 7800 × g for 15 min and the supernatant retained. The progress of endogenous peroxidation was followed by adding 1 mL 0.36% thiobarbituic acid (Sigma-T550-0) to 2 mL of supernatant. The mixture was boiled for 10 min, allowed to cool, then the production of lipid peroxide was measured by a Carry Conc. UV-Visible Spectrophotometer (Specord 250 plus, Analytikjena) and absorbance levels acquired by spectrometry were at 532 nm (adapted from Partyka et al., 2007).

Artificial Insemination

For fertility test, straws were thawed in an ice water bath at 5°C for 5 min. All hens (6 hens/each treatment) were inseminated once every 14 D with frozen/thawed semen from each group at a dose of 0.4 mL (mean 400 million sperm/female). AI was performed between 3:00 to 5.00 pm. Nine replications of the fertility test were carried out, and the females were allowed to different treatment for each replication. Eggs were collected on days 2 to 8 after each insemination before incubation.

Fertility was determined by candling eggs on day 7 of incubation. The fertility rates were calculated with the following formulas: (total number of fertile eggs/total number of incubated eggs) × 100.

Statistical Analysis

The experiment was conducted as a randomized complete block design. The results are presented as mean ± SD. Means were analyzed with ANOVA followed by Duncan's multiple range test to determine significant differences between groups. All percentage data were arcsine transformed before statistical analysis. A probability level of $P \leq 0.05$ was considered as significant.

RESULTS

Effect of a Large Range of Sucrose Doses on the Vigor Score and Membrane Integrity of Fresh and Frozen-thawed Chicken Sperm

The effects of 0 to 100 mmol of sucrose added to BHSV-based extender for unfrozen semen, or BHSV+ 6% DMF for frozen-thawed semen, are reported in Table 2. For the first sub-experiment, the presence of 0.5 to 0.75 mmol sucrose increased the vigor score of unfrozen semen ($P < 0.05$), but not the membrane integrity. In the second sub-experiment, 1 to 10 mmol sucrose increased the vigor, while the vigor score was altered only by the high doses of 75 and 100 mmol of sucrose. One mmol sucrose increased also the membrane integrity of unfrozen sperm but the doses higher than 10 mmol decreased the sperm membrane integrity ($P < 0.05$).

The effects observed on frozen/thawed semen were close to the effects on unfrozen semen but screened more the different doses. The doses of 0.5 to 10 mmol increased the sperm vigor and the doses 0.5 to 1 mM increased the membrane integrity ($P < 0.05$) when compared to the control. The highest doses (from 25 mmol for vigor, from 15 mmol for membrane integrity) were harmful ($P < 0.05$)

Effect of a Large Range of Raffinose Doses on the Vigor Score and Membrane Integrity of Fresh and Frozen-thawed Chicken Sperm

The effects of 0 to 100 mmol of raffinose added to BHSV-based extender for unfrozen semen and BHSV+6% DMF of frozen-thawed semen are reported in Table 3. The doses lower than 1 mmol had no effect on sperm vigor or membrane integrity of unfrozen semen, but 1 and 5 mmol of raffinose increased sperm vigor ($P < 0.05$). One to 100 mmol of raffinose significantly decreased the membrane integrity of fresh semen ($P < 0.05$).

On frozen semen, the doses of raffinose higher than 10 mmol significantly decreased the sperm vigor score ($P < 0.05$) and the doses 1 to 100 mmol significantly decreased the membrane integrity ($P < 0.05$).

Effect of Targeted Doses of Sucrose and Raffinose on Objective Motility Parameters of Frozen-thawed Semen

Table 4 shows the motility parameters analyzed by CASA of chicken semen frozen-thawed with different levels of sucrose and raffinose. Most of the CASA parameters were not significantly affected by doses of sucrose and raffinose except the 1 mmol raffinose that decreased MOT.

Effect of Targeted Doses of Sucrose and Raffinose on Membrane Integrity, Acrosome Integrity, High Mitochondria Function, Lipid Peroxide Production of Frozen-thawed Chicken Sperm

The effects of different levels of sucrose and raffinose on membrane integrity, acrosome integrity, high mitochondria function and levels of lipid peroxide production are shown in Table 5. One mmol sucrose showed higher membrane integrity, acrosome integrity, and mitochondrial potential of frozen-thawed sperm compared to the other treatments ($P < 0.05$). Raffinose did not improve membrane integrity, acrosome integrity, and mitochondria function. The sucrose and raffinose doses

Table 2. Effect of the addition of sucrose on chicken sperm characteristic of unfrozen and frozen-thawed semen.

Sucrose (mmol)		Vigor score (1–5)	Membrane integrity (%)
	0	4.30 ± 2.16^{b}	87.92 ± 3.25
	0.25	4.32 ± 2.54^{b}	87.55 ± 3.11
	0.5	4.47 ± 2.31^{a}	87.84 ± 3.47
	0.75	4.50 ± 2.51^{a}	87.42 ± 2.99
Unfrozen semen (incubated 24 h)	0	4.35 ± 2.16^{C,D}	88.68 ± 2.43^{B,C}
	1	4.72 ± 2.16^{A}	93.25 ± 4.06^{A}
	5	4.60 ± 2.09^{B}	88.91 ± 4.15^{B,C}
	10	4.59 ± 2.04^{B}	89.42 ± 3.04^{B}
	15	4.36 ± 2.08^{C,D}	83.07 ± 5.99^{D,E}
	20	4.35 ± 1.16^{C,D}	82.42 ± 3.05^{E}
	25	4.14 ± 2.13^{D}	80.32 ± 4.96^{E,F}
	50	4.08 ± 2.12^{D}	80.30 ± 4.35^{E,F}
	75	3.89 ± 2.52^{E}	78.42 ± 3.16^{F}
	100	3.71 ± 2.15^{E,F}	77.96 ± 2.89^{F}
Sucrose (mmol)	0	3.53 ± 2.43^{c}	44.04 ± 3.05^{c}
	0.25	3.61 ± 1.48^{c}	46.75 ± 4.21^{b}
	0.5	3.74 ± 2.65^{b}	47.52 ± 3.09^{a}
	0.75	3.81 ± 2.14^{a}	47.84 ± 4.95^{a}
Frozen-thawed semen	0	3.60 ± 2.18^{C,D}	48.58 ± 2.59^{B}
	1	4.04 ± 1.16^{A}	52.03 ± 4.34^{A}
	5	3.82 ± 2.09^{B}	48.33 ± 5.08^{B}
	10	3.78 ± 2.15^{B,C}	47.37 ± 5.05^{B,C}
	15	3.56 ± 3.07^{D,E}	46.22 ± 4.93^{C,D}
	20	3.53 ± 2.19^{D,E}	43.68 ± 4.49^{E}
	25	3.17 ± 2.13^{F}	43.62 ± 3.37^{E}
	50	3.11 ± 3.21^{F}	42.28 ± 2.87^{E}
	75	2.85 ± 2.88^{G}	38.56 ± 3.02^{F}
	100	2.76 ± 3.01^{G}	37.82 ± 3.45^{F}

The results are expressed as means ± standard deviations. N = 6. Different letters within columns, ^{a,c}or $^{A-F}$for a given experiment, indicate significant differences ($P < 0.05$).

tested did not affect the levels of lipid peroxide production measured by TBARS.

Effect of Targeted Doses of Sucrose and Raffinose on Fertility Obtained with Frozen-thawed Chicken Sperm

Results of fertility rate after insemination with frozen-thawed sperm are shown in Table 6. Fertility levels of the eggs collected from hens inseminated with frozen-thawed semen containing 1 mmol sucrose were higher (91%) than the control BHSV extender (86%) and that the other treatments ($P < 0.05$). The other doses of sucrose (5 and 10 mmol) and all the doses of raffinose tested (1, 5, 10 mmol) were deleterious to sperm when compared to the control.

DISCUSSION

The need for ex situ in vitro conservation of avian genetic resources is widely recognized (Fulton, 2006; Blesbois, 2011; Biscarini et al., 2015; Liu et al., 2018; Thélie et al., 2018), but the storage of semen collected from chicken rare breeds and/or pure lines in sperm cryobanks has so far been considered in few national programs for conservation of animal genetic resources (Blackburn, 2006; Blesbois et al., 2007, 2011). Recent studies confirmed the difficulty of obtaining cryopreserved chicken semen with a simple method without significant loss of fertility potential (Blesbois, 2012; Thélie et al., 2018). The use of complex methods or additives may be efficient in some cases (Chuaychu-noo et al., 2017; Thélie et al, 2018), but they may not be applied in a wide range of field conditions. In the present study, we show that a simple chicken sperm cryopreservation method is highly successful after the addition in the medium of an external cryoprotectant easily available and cheap, the sucrose, used at a weekly or inactive osmotically low concentration. We also clearly show that the 2 saccharides tested here (sucrose and raffinose) do not show at all the same effect at low concentration and that their osmotically active doses are harmful.

Different external cryoprotectants have already been tested to improve chicken sperm cryopreservation. PVP is actually one of the most popular external cryoprotectants used in fowls (Lake and Ravie, 1981; Tselutin et al., 1999; Blesbois et al., 2007; Rakha et al., 2017b; Thélie et al., 2018). It is used alone or associated with an internal cryoprotectant. Di- and trisaccharides are well known external cryoprotectants, mainly employed with success in the vitrification of mammalian cells and embryos (Eroglu et al., 2002; Kuwayama et al., 2005; Emeka 2015; Lestari et al., 2018). Sucrose, trehalose, or raffinose have been usually employed at concentrations (mean 500 mmol) that share a high osmotic effect in order to increase the dehydration of the cells

Table 3. Effect of the addition of raffinose on chicken sperm characteristic of unfrozen and frozen-thawed semen.

Raffinose (mmol)		Vigor score (1–5)	Membrane integrity (%)
	0	4.22 ± 3.89^{a}	84.44 ± 3.43^{a}
	0.25	4.29 ± 2.50^{a}	85.24 ± 2.45^{a}
	0.5	4.31 ± 3.45^{a}	84.36 ± 5.87^{a}
	0.75	4.33 ± 2.87^{a}	85.14 ± 4.22^{a}
Unfrozen semen (incubated 24 h)	0	4.30 ± 2.11^{B}	87.47 ± 2.85^{A}
	1	4.47 ± 2.04^{A}	85.92 ± 5.53^{B}
	5	4.38 ± 4.11^{B}	82.93 ± 6.80^{C}
	10	$4.18 \pm 2.14^{B,C}$	$81.95 \pm 4.64^{C,D}$
	15	$4.22 \pm 3.13^{B,C}$	80.56 ± 4.36^{D}
	20	4.15 ± 3.24^{C}	80.44 ± 4.26^{D}
	25	4.14 ± 4.13^{C}	$79.94 \pm 5.72^{D,E}$
	50	3.85 ± 3.12^{D}	77.25 ± 4.36^{E}
	75	$3.74 \pm 2.01^{D,E}$	74.47 ± 2.49^{F}
	100	3.61 ± 3.14^{E}	72.15 ± 3.15^{G}
Raffinose (mmol)	0	3.42 ± 2.01^{a}	45.09 ± 3.22^{a}
	0.25	3.45 ± 2.65^{a}	46.31 ± 4.25^{a}
	0.5	3.49 ± 4.26^{a}	45.20 ± 3.87^{a}
	0.75	3.55 ± 3.25^{a}	45.67 ± 4.88^{a}
Frozen-thawed semen	0	$3.58 \pm 2.32^{A,B}$	47.56 ± 4.31^{A}
	1	3.63 ± 2.10^{A}	45.81 ± 4.45^{B}
	5	$3.51 \pm 3.10^{A,B}$	$44.20 \pm 5.96^{B,C}$
	10	$3.49 \pm 3.07^{B,C}$	43.60 ± 5.62^{C}
	15	3.30 ± 4.25^{C}	40.96 ± 4.22^{D}
	20	3.14 ± 4.20^{D}	39.62 ± 4.26^{D}
	25	3.08 ± 2.07^{D}	39.12 ± 3.99^{D}
	50	2.75 ± 2.66^{E}	32.21 ± 2.54^{F}
	75	$2.64 \pm 2.45^{E,F}$	31.87 ± 3.54^{F}
	100	2.51 ± 3.63^{F}	31.45 ± 4.35^{F}

The results are expressed as means ± standard deviations. N = 6. Different letters within columns, ^{a,b}or $^{A-F}$for a given experiment, indicate significant differences ($P < 0.05$).

Table 4. Percentage of motile sperm, progressive motile sperm, average path velocity, and straight-line velocity of semen cryopreserved with different concentrations of sucrose and raffinose.

Factor		MOT (%)	PMOT (%)	VAP (μm/s)	VSL (μm/s)
Control	0	$60.25 \pm 3.86^{a,b}$	24.83 ± 2.65	$64.05 \pm 3.07^{a,b,c}$	47.76 ± 2.21
Sucrose (mmol)	1	64.25 ± 4.53^{a}	25.41 ± 2.93	67.50 ± 4.49^{a}	49.38 ± 5.61
	5	$59.66 \pm 5.39^{b,c}$	24.58 ± 3.89	$63.46 \pm 3.16^{a,b,c}$	48.67 ± 2.17
	10	61.33 ± 4.79^{b}	25.25 ± 2.22	$66.25 \pm 9.87^{a,b}$	46.54 ± 3.93
Raffinose (mmol)	1	55.66 ± 2.74^{c}	23.75 ± 2.83	60.38 ± 3.38^{c}	45.50 ± 3.31
	5	$59.16 \pm 5.90^{b,c}$	24.16 ± 3.64	62.08 ± 2.71^{a}	50.22 ± 5.23
	10	$60.33 \pm 5.28^{a,b}$	24.41 ± 2.44	$64.78 \pm 5.24^{a,b,c}$	48.68 ± 3.98

The results are expressed as means ± standard deviations. N = 9. Different letters (^{a,b}) within columns indicate significant differences ($P < 0.05$), MOT (%): percentage of motile spermatozoa; PMOT (%): percentage of spermatozoa with progressive motility; VAP (μm/s): percentage of velocity average path; VSL (μm/s): percentage of velocity straight line.

during freezing; and in embryos, to decrease the osmotic shocks at thawing. In mammalian sperm, different studies reported that disaccharides provide high protection of sperm by increasing motility, plasma membrane integrity, and fertilizing capacity of post-thawed sperm in mouse and deer (Tada et al., 1990; Yokoyama et al., 1990; Gadre et al., 2008). Although several substances have been used as cryoprotectants, the disaccharide stands out as particularly excellent for a wide range of biologically important storage properties, such as a high recovery of protein functions after cryostorage and an excellent ability to protect lipid bilayers to low and increasing temperatures (freeze-thaw) (Crowe, 2002; Chiantia et al., 2005). Trisaccharides may also be efficient: 5 mmol raffinose increased cryoprotection and post-thaw semen quality (sperm motility parameters, acrosome integrity, high mitochondria activity, and viability) in goat (Tuncer et al., 2010), bull (Tuncer et al., 2011), and ram (Bucak et al., 2013). In avian species, disaccharides showed contrasted effects on the success of sperm cryopreservation (Blanco et al., 2011; Mosca et al., 2016; Miranda et al., 2017; Brown et al., 2018). The use of 100 to 200 mmol of sucrose as a non-permeant cryoprotectant did not protect cryopreserved crane sperm and induced low post-thawed survival and ability to bind to the inner perivitelline

Table 5. Effects of different concentrations of sucrose and raffinose on membrane permeability, acrosome integrity, functional mitochondria, and malondiodehyde of cryopreserved chicken sperm.

Factor		Membrane integrity (%)	Acrosome integrity (%)	Functional mitochondria (%)	MDA (μm/mL/150 $\times$ 10^6 spz)
Control	0	39.96 ± 3.39^{d}	35.11 ± 2.95^{b}	49.09 ± 4.65^{b,c}	2.02 ± 0.33
Sucrose (mmol)	1	54.73 ± 3.06^{a}	42.96 ± 4.66^{a}	55.89 ± 3.66^{a}	2.00 ± 0.39
	5	48.39 ± 3.66^{b}	34.34 ± 3.61^{b}	50.36 ± 2.40^{b}	1.95 ± 0.24
	10	46.31 ± 4.26^{b,c}	34.40 ± 2.19^{b}	49.40 ± 2.87^{b,c}	1.90 ± 0.18
Raffinose (mmol)	1	45.35 ± 2.89^{b,c}	32.99 ± 2.22^{b}	48.91 ± 3.38^{b,c}	1.80 ± 0.18
	5	44.59 ± 3.11^{b,c}	32.23 ± 4.58^{b}	46.63 ± 2.76^{d,c}	1.98 ± 0.23
	10	43.05 ± 2.82^{d,c}	33.18 ± 3.76^{b}	45.05 ± 3.81^{d}	1.98 ± 0.14

The results are expressed as means ± standard deviations. N = 9. Different letters (^{a,b}) within columns indicate significant differences ($P < 0.05$), MDA; malondialdehyde.

Table 6. Effects of different concentrations of sucrose and raffinose on fertility capacity of cryopreserved chicken sperm.

Factor		No. fertile eggs	No. incubated eggs	Fertility (% fertile/ incubated eggs)
Control	0	241	279	86.1 ± 2.77^{b}
Sucrose (mmol)	1	254	278	91.16 ± 2.39^{a}
	5	227	280	80.92 ± 4.57^{c}
	10	228	287	79.32 ± 3.40^{c}
Raffinose (mmol)	1	204	289	70.38 ± 4.47^{d}
	5	184	277	66.38 ± 5.77^{d}
	10	181	273	66.01 ± 4.76^{d}

The results are expressed as means ± standard deviations. N = 9. Different letters (^{a,b}) within columns indicate significant differences ($P < 0.001$).

membrane (Brown et al., 2018). Miranda et al. (2017) reported that 400 mmol sucrose decreased chicken semen motility after cryopreservation.

In the present work, we show that a much lower concentration of sucrose (1 mmol) is efficient in order to increase the sperm plasma membrane integrity/viability, acrosome integrity, mitochondrial potential, and finally the fertility obtained with frozen-thawed chicken sperm. Higher doses were less and less efficient, and finally, the highest and osmotically active doses tested (from 15 to 100 mmol) were all harmful to sperm. The filiform shape of bird sperm induces specific properties (reviewed by Blesbois and Brillard, 2007; Bakst, 2014). Since avian sperm contain relatively low proportions of intracellular water when compared to most diploid cells and embryos, and, in many cases to mammal sperm, we could suggest that it is not useful to purchase a high level of dehydration during the chicken sperm freezing process, and so that high concentrations of external cryoprotectants are not needed. We could also suggest that a great and not needed osmotic effect of high external cryoprotectant concentrations that leads to aggressive dehydration is deleterious to the sperm membranes. This would explain the harmful effect of 15 to 100 mmol sucrose.

The positive effect of low doses of sucrose would much probably be explained by not osmotic actions. A membrane coating effect of disaccharides has previously been suggested (Brandley and Schnaar, 1986; Storey et al., 1998; Olsson et al., 2016). This coating may include strong covalent interactions or electrical van der Waals interactions with the sperm membranes components (Patist and Zoerb, 2005). These factors may increase the stability of the sperm membranes and explain the positive action of low sucrose doses on the sperm membrane integrity, the acrosome integrity, the mitochondrial functions and consequently the sperm fertilizing ability in our present study.

The trisaccharide raffinose was much less efficient than the disaccharide sucrose in our study. If 1 mmol raffinose showed an increase in the motility vigor score, it did not improve sperm membrane integrity, acrosome integrity, mitochondrial functions and finally it showed a negative effect on fertility by contrast to 1 mmol sucrose. The other doses tested were no more efficient and the highest doses, osmotically active, were clearly harmful. The composition of sucrose and raffinose is not so different. Sucrose is composed of glucose and fructose while raffinose is composed of sucrose plus 1 molecule of galactose. Raffinose and sucrose show an osmotic effect approximately at the same doses (Table 1) that may not explain the differences of results obtained in the present study between the 2 saccharides. If we have a look on the potential coating effect of these 2 molecules in order to protect sperm membranes, we may suggest that it could differ between a di- and a trisaccharide because of their difference of size, but this is speculative at this stage. Another question would be the potential role of galactose included in the raffinose but not in the sucrose.

Galactose is a sugar that may share many actions on the metabolism of the cells and on it senescence. Previous studies on specific somatic or reproductive cells indicate an effect of galactose on the acceleration of apoptosis, oxidative alterations (Thakur et al., 2017), and inhibition of sperm-egg binding in the mouse (Shur and Hall, 1982). Some of these functions are not specific to galactose and may also be attributed to other sugars. However, in the present study, we could suspect that galactose that is the only sugar that differs between sucrose and fructose, could be involved in the lower results obtained with raffinose than with sucrose.

In our study, the fertility rates obtained with frozen semen are comparatively higher than in many chicken studies. In our study, fertility was measured on the result of once a week inseminations at the difference of the practice in other chickens and other species, where twice or thrice a week inseminations are required to get good fertility with cryopreserved sperm (Blanco et al., 2012; Partyka et al., 2012; Santiago-Moreno et al., 2012; Thélie et al. 2018). In addition, a simple freezing curve of freezing, without programmable freezer, and a simple thawing process without centrifugation were used here. The based extender is the BHSV combined with the internal cryoprotectant DMF. This combination is considered now as the best alternative to the combination Lake-extender and glycerol cryoprotectant that is the most successful combination in laboratory conditions (Thélie et al., 2018). Interestingly, the BHSV as well as the Lake extender are hypertonic to seminal plasma. The success of these hypertonic extenders compared to seminal plasma (401 to 420 mOsm/Kg vs. 305 to 338 mOsm/Kg for Thai native chicken seminal plasma, Thananurak et al., 2017) is in accordance with previous assumptions. Indeed, many authors (Lake, 1984; Sakhatsky, 1990; Blesbois and Brillard, 2007; Getachew, 2016) stated that the specific constituents of extenders are less important than the overall pH and osmotic pressure, and that the last should be a little hypertonic to seminal plasma (by 50 and 100 mOsm/kg). We must also remember that the fertility success after sperm cryopreservation is multifunctional. In addition to the methodologic points discussed here, we must remember that the initial semen quality is a key factor (Blesbois et al., 2008). The conditions of life of the animals are also important to ensure peaceful behaviors and good cooperation between the male fowls and the humans that are involved in their breeding and in the sampling of semen.

In conclusion, the success of an efficient utilization of semen cryopreservation is multifactorial. In the chicken, it must combine a careful breeding of the animals, a specific attention to their welfare, a good initial semen quality, and an efficient method of cryopreservation. The present study shows that sperm cryopreservation of a Thai local breed raised in careful and good conditions is highly successfully developed by the use of a combination of a simple freeze-thaw process and the addition of low sucrose concentration. We recommend the presence of 1 mmol sucrose concentration in the freezing extender in addition to an internal cryoprotectant in order to increase chicken semen freezability.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

ACKNOWLEDGMENTS

This study was funded by Thailand Research Fund (TRF) under Research and Researcher for Industries (RRI) (PHD59I0025), by the European Union's Horizon H2020 Research and innovative Program IMAGE under the grant number 677353 and the French National Institute of Agronomic Research. The authors would like to thank the Research and Development Network Center for Animal Breeding (Native Chicken) and the Department of Animal Science, Faculty of Agriculture, Khon Kaen University, for supporting animal experiments. We thank Isabelle Grasseau for her detailed and instruction in the technique for semen quality evaluation. We are also grateful to the Institute of Agricultural Technology, Suranaree University of Technology, Northeast Agriculture Research Center, Faculty of Agriculture, Khon Kaen University for semen quality evaluation equipment.

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