Original Article

Cryopreservation methods of Thai native chicken (Pradu-hangdum) semen: effect of different freezing containers on motion and kinetic parameters of frozen-thawed sperm

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Abstract

Artificial Insemination (AI) programs using qualified semen are designed to revolutionize the poultry farming sector by targeting genetic improvement along with appropriate health strategies. Hence, a prolonged preservation method of quality sperm conserves is important for successful AI outcomes. The objective of this study was to compare the effects of different type of containers; straw, cryovial tubes and pellet on the quality of the frozen-thawed Thai native chicken (Pradu-hangdum) semen. High-quality pooled semen was collected and divided into 4 groups with 12 replicates: semen preserved with BHSV extender in 1) 0.25 mL straw, 2) 0.5 mL straw, 3) 2 mL cryovial tube, and 4) semen pellet in 2 mL cryovial tube. All containers were stored in a liquid nitrogen tank for 1 week and the frozen-thawed sperm were subsequently analyzed. The results indicated that the mean of all motion parameters including total motility, progressive motility, progressive fast motility and progressive slow motility, and some kinetic parameters such as DCL, DSL, ALH, BCF, and HAC of the frozen-thawed sperm preserved in cryovial tube had significantly higher values compared to other groups ($P < 0.05$). In conclusion, semen freezing process in cryovial tubes had the least effect on decreasing the qualities of frozen-thawed sperm than other methods. Furthermore, fertility rates related to chicken semen preservation should be further evaluated to provide valuable knowledge to farmers.

Keywords: cryovial tube, freezing process, frozen-thawed sperm quality, pellet, Pradu-hangdum chicken, straw

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Introduction

The poultry sector has recently become a major contributor to Thailand's economy as a result of the revolutionary scientific approaches in chicken health care and efficient breeding management. The Thai government has invested in the development of strategies to provide pure poultry genetics to farmers to increase meat production (Leotaragul & Kammongkun, 2010). However, the major problems of natural insemination that result in the low chicken population were the low rates of fertility due to the ratio of male to female chicken and crossbreeding. AI is well-known as an attractive breeding program for increasing mating ratio in distinctive chicken species and in propagating the desirable characteristics of one male to many progenies resulting in genetic improvement (Dhama *et al.*, 2014).

Long term preservation methods have been developed for the conservation of avian sperm reproductive potential. Cryopreservation is known to be highly stressful for the sperm leading to decreased rates of fertilization. During the process, osmotic, thermal shocks and toxic effects might cause significant damage to cell structure and metabolism (J. Long, 2006). Slow and rapid freezing are the conventional cryopreservation methods. Slow freezing is a method in which sperm cells are cooled progressively over a period of 2-4 h in two or three steps. It has been suggested that it induces possible chemical-physical damages to the spermatozoa (Di Santo, Tarozzi, Nadalini, & Borini, 2012). Cooling rates must be controlled at a certain value to reduce osmotic injury (Said, Gaglani, & Agarwal, 2010). In the rapid freezing technique, spermatozoa are mixed with the cryoprotectant and the mixture is loaded into the straw or cryovial tube and subsequently exposed to a liquid nitrogen vapor phase for at least 10 min before being sunk into liquid nitrogen (Di Santo *et al.*, 2012). Several studies have compared slow and rapid freezing methods and reported that sperm frozen through rapid freezing methods had significantly better qualities than through slow freezing methods (Tongdee, Sukprasert, Satirapod, Wongkularb, & Choktanasiri, 2015; Váradi, Végi, Liptói, & Barna, 2013; Vutyavanich, Piromlertamorn, & Nunta, 2010). However, previous studies also indicated that the freezing process affects to decrease sperm motility and the ability of the sperm to acrosomal reaction (Mocé, Grasseau, & Blesbois, 2010). One of the keys to achieving high fertility rates is high sperm motility after preservation. However, in chicken AI, there were limited studies on the effects of freezing methods on sperm motility (J. A. Long, 2006; Long *et al.*, 2014).

Nowadays, many cryotube products can be used for sperm cryopreservation. Straw has been used in many mammals due to its straws as a packaging system have benefits such as thin plastic surface, sperm traceability and appropriateness with the female reproductive anatomy during insemination(Iaffaldano *et al.*, 2016). However, the primary target sites of sperm insemination in chicken are the sperm storage tubules (SSTs) (Das, Isobe, & Yoshimura, 2008). SSTs, where sperm can be stored and can survive even after insemination or natural mating, are located in the uterovaginal junction of the oviduct, approximately 3-4 cm from the vaginal orifice (Bakst & Akuffo, 2009; Sasanami, Matsuzaki, Mizushima, & Hiyama, 2013). Because of this unique avian anatomy, there is no need for long tubes or insertion tools in the chicken insemination technique. Among the freezing systems of avian spermatozoa, the pellet procedure is cheap, easily adaptable to field conditions, takes only a few seconds for cooling and warming, the cryoprotectant does not have to be removed (Tselutin, Seigneurin, & Blesbois, 1999). Cryovial tubes have been widely used in human sperm preservation due to its safety from cross-contamination. It has been reported that human sperm preserved in cryovial tubes have higher motility values than those preserved in straw (Li *et al.*, 2019). This fact emphasizes that cryovial tubes could be a candidate choice for avian sperm cryopreservation. However, there were no previous reports on the effects of the type of freezing container on the qualities of frozen-thawed chicken sperm. To provide new knowledge on this, the objective of the present study was to compare 4 different cryopreservation methods of Pradu-hangdam semen using straw and cryovial tubes with focus on the sperm motion and kinetic values.

Materials and Methods

Animals: Thirty, one- year- old purebred Pradu-hangdum roosters having the phenotypic characters described by (Kammongkun & Leotaragul, 2015) and weighing an average of 3.5 kg were randomly selected. All chickens were housed individually in a high-rising cage (120 cm ×120 cm ×120 cm). The cages were exposed to natural light for 12 h per day. 17% protein-containing commercial feeds were provided to the chicken at an approximately 200 grams/chicken/day with water supplied ad libitum. All chickens were trained for semen collection through dorso-abdominal massage for at least 1 month before the start of the first semen collection (Burrows & Quinn, 1937). All experiments were conducted in accordance with the Institutional Animal Care and Use Committee guidelines of the Faculty of Veterinary Medicine, Chiang Mai University under the approved protocol number S33/2561.

Semen collection: Semen was collected in the morning using dorso-abdominal massage. Briefly, each chicken was held up with its tail lifted to prevent semen contamination. The slow soft massage continued from the dorsal part of the back to the ventral abdominal area until cloacal protrusion occurred. The milky-colored semen was collected in a 1.5 mL sterile plastic tube (Eppendorf, Humburg, Germany), subsequently stored with BHSVextender and kept at 4 °C until analysis.

Fresh Semen analysis: Semen quality was analyzed by macroscopic and microscopic examination under the light microscope. Good quality semen was collected

following these criteria: white cream color, sperm concentration > 4.0 ×10^9 cells per mL, mass motility > 4 (4/5 score), total motility > 80% (Sonseeda, Vongpralub, & Laopaiboon, 2013). Semen was pooled and kept at 4 ^oC until used.

Freezing semen preparation: The pooled semen was diluted with the Blumberger Hahnen Sperma Verdünner (BHSV) extender (glucose 0.5 g, sodium glutamate 2.85 g, potassium acetate 0.5 g, magnesium acetate 0.07 g, myo-inosital 0.25 g and ultrapure water 100 mL) with 7.15 pH and 380 mOsm osmolarity and kept at 4 ^oC for 5 min (Seigneurin, Grasseau, Chapuis, & Blesbois, 2013). 10 % (v/v) ethylene glycol (EG) was added into the semen extender and mixed with the semen. The mixture semen of final concentration 0.8-1.0 ×10^9 sperms per mL was equilibrated for 30 min at 4 ^oC.

Semen was divided into 4 groups, each containing 12 replicates. The first and the second semen groups were prepared in 0.25 mL and 0.5 mL straw tubes (Minitube GmbH, Tiefenbach, Germany) for the freezing process, respectively. The semen mixture was gently inserted in each straw tube using an auto-pipette. The straws were placed at approximately -130 ^oC (3 cm over the liquid nitrogen) for 10 min and then kept in liquid nitrogen. The third group was prepared by transferring 0.5 mL semen into a 2 mL cryovial tube (Biologix Group Limited, Shandong, China) and the tube was horizontal placed at approximately -130 ^oC (3 cm over the liquid nitrogen) for 10 min and kept in liquid nitrogen. The last group was prepared as 50 µL pellet (0.5 mL) and preserved in 2 mL cryovial tube for the freezing process. The pellets were placed on a glass plate at approximately -130 ^oC (3 cm over the liquid nitrogen) for 10 min and kept in liquid nitrogen for 1 week.

Post-thawed sperm examination: Frozen semen was separately thawed at 40 ^oC for 15 sec, 30 sec and 1 min before analysis. Post-thaw sperm motion and kinetic parameters were evaluated using computer-assisted semen analysis (CASA, Hamilton Thorn Motility Analyzer, IVOS12.3, USA). The analysis setup used to evaluate the chicken sperm and using a Leja slide, 3µl of the prepared semen sample were loaded and analyzed on the CASA machine with many microscopic fields each, recording at least 1,000 sperms. The semen samples were analyzed through CASA based on the travelled distance, velocity, head movement, and trajectory. The measured sperm kinematics were average distance path (DAP, µm), distance curved line (DCL, µm), distance straight line (DSL, µm), average path velocity (VAP, µm), curvilinear velocity (VCL, µm), straight line velocity (VSL, µm), amplitude of lateral head movement (ALH, µm), beat cross frequency (Hz), linearity (LIN, %), straightness (STR, %) and wobble (WOB, %).

Statistical analysis: The mean and standard error of the mean (SEM) of motion parameters and sperm kinetics were described and compared by one-way ANOVA and student T-test using Graphpad Prism 8.3.1 (Graphpad Software Inc., California, USA). The significant difference was accepted at the *P*-value < 0.05.

Results

Fresh semen evaluation: All 30 fresh semen specimens presented a white cream color and passed the minimum concentration criteria. The mean sperm motility was 92.42 ± 6.82%. But only 21 out of 30 (70 %) semen specimens passed the mass motility and total motility criteria. Therefore, the pooled semen in this study was prepared from 21 high- quality semen specimens.

Effects of different freezing process on sperm motion parameters: The motion parameter results of the different freezing processes of semen preservation were shown in Table 1. The means of all motion parameters (total motility, progressive motility, progressive fast motility, and progressive slow motility) of frozen-thawed sperm obtained from semen preserved in cryovial tube (64.31%, 39.45%,14.65% and 24.79%, respectively) were significantly higher those from the other groups ($P < 0.05$). Likewise, the means of total motility, progressive motility, and progressive fast motility of frozen-thawed sperm obtained from pellet preserved in cryovial tube (53.34%, 30.24% and 18.27%) were also significant higher than from sperm preserved in 0.25 mL (53.09, 11.60% and 18.34%) and 0.5 mL straws (47.70%, 10.66% and 18.34%), respectively ($P<0.05$). Despite this, the mean progressive fast motility of frozen-thawed sperm obtained from pellet preparation (11.96%) was a bit higher than from that of sperm preserved in 0.25 mL straw (11.60%) but significant higher than from the sperm preserved in 0.5 mL straw (10.66%) ($P<0.05$).

Table 1 Motion parameters of frozen-thawed Pradu-hangdam chicken semen using different freezing methods

Freezing methods	TM (%)	PM (%)	PFM (%)	PSM (%)
0.25 Straw	53.09 ± 2.29 ^{a,b}	29.94 ± 1.84 ^{a}	11.60 ± 1.15 ^{a,b}	18.34 ± 1.05 ^{a}
0.5 Straw	47.70 ± 2.30 ^{a,b}	27.29 ± 1.67 ^{a}	10.66 ± 0.54 ^{a}	16.63 ± 1.24 ^{a}
Cryotube	64.31 ± 3.04 ^{c}	39.45± 2.45 ^{b}	14.65 ± 1.17 ^{b}	24.79 ± 1.73 ^{b}
Pellet	53.34 ± 2.67 ^{b,c}	30.24 ± 2.27 ^{a}	11.96 ± 0.90 ^{a,b}	18.27 ± 1.54 ^{a}

TM= Total motility, PM= Progressive motility, PFM= Progressive fast motility, and PSM= Progressive slow motility.
Mean and SEM with different superscript letters indicate significant differences (*P*-value < 0.05).

Effects of different freezing process on sperm kinetic parameters: The kinetic parameter results of the different freezing processes of semen preservation were shown in Table 2. The distance average path (DAP), distant straight line (DSL), amplitude of lateral head movement (ALH), beat-cross frequency (BCF), and head activity (HAC) of frozen-thawed sperm obtained from semen preserved in cryovial tube (5.63 μm/s, 2.99 μm/s, 0.68 μm/s, 5.39 μm/s, and 0.21 μm/s, respectively) were significantly higher than those from the other groups (4.44 μm/s, 2.43 μm/s, 0.54 μm/s, 3.85 μm/s, and 0.17 μm/s, respectively) ($P < 0.05$). In contrast, there was no significant difference in velocity straight line (VSL), distance curved line (DCL), wobble (WOB), linearity (LIN), and straightness (STR) kinetic parameters among the 4 different freezing containers.

Table 2 Kinetic parameters of frozen-thawed Pradu-hangdam chicken semen using different freezing methods

Freezing methods	VCL (μm/s)	VSL (μm/s)	VAP(μm/s)	DCL (μm/s)	DSL(μm/s)	DAP(μm/s)
0.25 Straw	54.65 ± 2.63 ^{a,b}	21.43± 1.82	27.08 ± 1.84 ^{a,b}	13.55 ± 0.87	2.44 ± 0.11 ^{a}	4.55 ± 0.23 ^{a}
0.5 Straw	51.52 ± 1.86 ^{a}	20.70 ± 1.09	25.84 ± 1.04 ^{a}	12.60 ± 0.97	2.32 ± 0.11 ^{a}	4.22 ± 0.29 ^{a}
Cryotube	63.19 ± 3.93 ^{b}	24.48 ± 1.54	31.90 ± 1.51 ^{b}	15.94 ± 1.29	2.99 ± 0.13 ^{b}	5.63 ± 0.35 ^{b}
Pellet	55.30 ± 2.88 ^{a,b}	22.36 ± 1.38	28.01 ± 1.50 ^{a,b}	13.26 ± 0.85	2.53 ± 0.13 ^{a}	4.56 ± 0.25 ^{a}

VCL=Velocity curve line, VSL=Velocity straight line, VAP=Average path velocity, DCL= Distance curve line, DSL= Distance straight line, and DAP=Distance average path

Mean and SEM with different superscript letters indicate significant differences (P-value < 0.05).

Table 2 (cont.) Kinetic parameters of frozen-thawed Pradu-hangdam chicken semen using different freezing methods

Freezing methods	ALH (μm/s)	BCF (μm/s)	HAC (μm/s)	WOB (μm/s)	LIN (μm/s)	STR(μm/s)
0.25 Straw	0.56 ± 0.02 ^{a}	3.94± 0.31 ^{a}	0.18 ± 0.01 ^{a}	0.49 ± 0.02	0.39 ± 0.02	0.78 ± 0.02
0.5 Straw	0.52 ± 0.03 ^{a}	3.65 ± 0.31 ^{a}	0.16 ± 0.01 ^{a}	0.50 ± 0.02	0.40 ± 0.02	0.80 ± 0.02
Cryotube	0.68 ± 0.04 ^{b}	5.39 ± 0.43 ^{b}	0.21 ± 0.01 ^{b}	0.49 ± 0.02	0.38 ± 0.02	0.76 ± 0.02
Pellet	0.56 ± 0.03 ^{a}	3.97 ± 0.28 ^{a}	0.18 ± 0.01 ^{a}	0.51 ± 0.01	0.41 ± 0.02	0.80 ± 0.01

ALH= amplitude of lateral head movement, BCF= Beat cross-frequency, HAC= Head activity, WOB= wobble, LIN= Linearity, and STR=Straightness

Mean and SEM with different superscript letters indicate significant differences (P-value < 0.05).

Discussion

Evaluation of fresh semen before storage is important for providing an estimate of semen quality. In general, the qualities of semen after prolonged storage will be decreased by several factors (Iaffaldano, Romagnoli, Manchisi, & Rosato, 2011; Miranda *et al.*, 2018; Mosca *et al.*, 2016; Olexikova, Miranda, Kulikova, Baláži, & Chrenek, 2019). Similarly, the motility parameter of sperm in this study also decreased from its originating fresh semen (Duplaix & Sexton, 1984). but the post-thaw sperm motility remained within the range of acceptable values for insemination (i.e. 40–50%) (Surai & Wishart, 1996). However, the low motility of frozen-thawed sperm had strong correlations between the percentage of immotile sperm and mitochondrial defects after thawing (Ozkavukcu, Erdemli, Isik, Oztuna, & Karahuseyinoglu, 2008). Additionally, the rapid changes in osmolarity and intracellular ice crystal formation during the cryopreservation process that lead to membrane structure disruption and DNA damage were also suggested (Gliozzi, Zaniboni, & Cerolini, 2011; Pedersen & Lebech, 1971). Thus, high-quality fresh semen was prepared for prolonged preservation.

The cooling rate is an important crisis point of the freezing process and it may affect frozen-thawed sperm quality after prolonged preservation. A suitable container at each freezing process protocol needs to be evaluated. In our rapid protocol, the motility of chicken sperm preserved in the cryovial tube was significantly higher than the others preserved in the straw tube. These results were similar to a previous study in frozen-thawed human sperm cryopreserved in cryovial tube (Li *et al.*, 2019). The shape of the cryovial tube may be one of the several factors that affected the rate of the temperature change inside the tube during cooling and warming (Hasegawa, Yonezawa, Ohta, Mochida, & Ogura, 2012). The plastic straw tube is thinner than the cryotube which may also result in too fast cooling and warming rates. These then suggest that the cooling rate should be optimized for semen freezing protocols. Because of the unique morphology of avian spermatozoa, such as filiform head shape, and long tail, which makes them more susceptible to freezing damage (Donoghue & Wishart, 2000; J. A. Long, 2006). However, the results of the present study were in contrast to the previous ones. They showed that the types of tubes had no effects on the fertility rate and that the use of plastic straw indicated shorter storage duration after fertility than the cryovial tube (Latorre, Harris, & Johnson, 1988). Probably, our protocol is best suited for conical-bottom cryotube than other containers.

Pellet formation has been an attractive, new, rapid-freezing method. As verified by many scientists, the fertility rate of frozen-thawed sperm preserved using this method was higher than in the straw preparation method (Surai & Wishart, 1996; Woelders, Zuidberg, & Hiemstra, 2006). In this study, the quality (both motion and kinetic parameters) of sperm in pellet formation tends to be higher than in the straw method (though not significant), with our data on percentage motility being the same as Shanmugam and Mahapatra (2019).

Within the same cryopreservation tube, sperm preserved using pellet formation had lower sperm motility and kinetic parameters. This could be explained in terms of the surface of sperm contact with the environment. Pellet formation increases the ability of sperm contact with a cooling surface (glass plate) which then leads to increased cooling rates as compared with the non-pellet methods. The cooling rate could affect the viability of chicken sperm (Blanco, Gee, Wildt, & Donoghue, 2000). Hence, a high cooling rate might be one of the potential explanations of the decreased quality of the frozen-thawed sperm in the present study (Hasegawa *et al.*, 2012).

Moreover, the different sizes of straw have no significant effects to the sperm motility, similar to the findings of a previous study which presented that the size of straw did not affect the fertility of chicken (Duplaix & Sexton, 1984). In a study where semen was preserved in straws, the temperature at approximately 3 cm above the liquid nitrogen vapor decreased quickly at around 30 ºC/ min in the initial two minutes, subsequently followed by a constant decrease until the final temperature (Madeddu *et al.*, 2016). These 2 sizes of straws (0.25 and 0.5 mL) reached the final temperature at the same time. During freezing in the liquid nitrogen, the semen preserved in 0.25 mL straw tube has a higher opportunity to contact the liquid nitrogen more than that of the 0.5 mL. So, the optimal cooling rate of the 0.25 mL straw was better than 0.5 mL straw.

The reduction of sperm motion and kinetic parameters after cryopreservation is an important topic of the current research. Some sperm kinetic parameters such as DAP, DSL, ALH, BCF and HAC were correlated to the motion parameters in cryovial tube-cryopreserved group. As with the data of Peña, Johannisson, Wallgren, and Rodríguez- Martínez (2003), frozen-thawed sperm was characterized by an increase in LIN and a decrease in VCL and ALH. However, VSL and VAP did not increase after the frozen-thawed procedures. Vutyavanich *et al.* (2010) reported the cryopreservation techniques caused a change in sperm kinetics or severe loss of sperm motility. The most sensitive parameters were the percentage of progressive motile sperm, VAP, VSL and VCL, whereas ALH and LIN were unchanged (Bolten, Weissbach, & Kaden, 2005). Moreover, Muiño, Tamargo, Hidalgo, and Peña (2008) explained that the ejaculated semen that is most resistant to cryopreservation showed the best post-thaw sperm longevity and contained the highest populations of rapid and progressive sperm. Furthermore, based on the findings of Leão Freitas, Silva Bouéres, Gonçalves de Oliveira, de Oliveira Viu, and Arruda de Oliveira (2015) and Muiño *et al.* (2008), greater values of VAP, VSL, VCL, and ALH obtained at post-thaw indicate a hyperactive movement of the spermatozoa, thereby giving a higher rate of pregnancy.

In conclusion, this study highlighted that Pradu-hangdum chicken sperm preserved in cryovial tube with BHSV- EG extender had the least effect on decreasing frozen-thawed sperm qualities as compared with the other groups. Moreover, the freezing technique in this study could be possibly applied to other avian species.

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