Original Article

Effect of modified smart extender on sperm longevity and frozen-thawed semen quality in Bantam chicken (*Gallus gallus*)

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Abstract

The round body shape and short legs of Bantam chickens (*Gallus gallus*) pose breeding challenges, making artificial insemination critical for these animals. To contribute to such assisted reproductive technology in avians, this study assessed the effect of a modified simple medium for assisted reproductive technology (SMART) extender on sperm longevity and frozen-thawed semen quality compared to the Blumberger Hahnen Sperma Verdünner (BHSV) extender. Sperm motility and viability were measured in 20 roosters at fresh and chilled conditions at 4-, 24-, and 48-hour intervals. For the subsequent freezing step, 6% and 9% DMA were added to both extenders as cryoprotectants. Results showed significant declines in sperm motility and viability over time (P<0.05) using the modified SMART extender, indicating that the extended semen should be used within 4 hours of collection. CASA analysis also found no significant differences in total motility between the different combinations of extenders and cryoprotectant levels (P>0.05). However, notable differences in progressive motility were observed between the modified SMART extender with 6% DMA and the BHSV extender with 6% and 9% DMA (P<0.05). In conclusion, the modified SMART extender effectively extends semen longevity but falls short of the BHSV extender in terms of cryopreservation efficiency.

Keywords: Bantam chicken, Modified SMART extender, Longevity, Freezability

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Introduction

The Bantam chicken (*Gallus gallus*), a miniature breed descended from the red jungle fowl, is known for its distinctive overall teardrop appearance. It is characterized by its colorful plumage, short legs, short back, round chest, and high, well-carried tail (Chuaychu-noo *et al.,* 2021; Bureau of Animal Husbandry and Genetic Improvement, 2002). These unique attributes of Bantam chickens have attracted considerable attention among the avian community and poultry enthusiasts worldwide. In Thailand, it has become one of the most popular chicken breeds among pet owners, driving the growth and expansion of specialized breeding farms in the country in recent years. However, these farms face significant breeding challenges related to Bantam's body shape and short legs, which, in turn, hinder natural reproductive processes (Chuaychu-noo *et al.,* 2021). In addition, the presence of the semi-lethal, dominant Creeper gene (Cp) associated with the 'short legs' trait increases the risk of offspring loss (Jin *et al.,* 2016). To address this issue of carrying the double Cp gene, Bantam chickens are usually bred with non-carrier chickens or long-legged varieties (Wang *et al.,* 2017). Yet, short-legged offspring are still preferred over their long-legged counterparts. To overcome the aforementioned challenges and ensure a higher yield of short-legged offspring, artificial insemination has emerged as a valuable strategy for Bantam chicken breeding and selection.

Semen quality is paramount to successful artificial insemination, with extenders being essential to the process. They act as energy sources for sperm metabolism and regulate semen pH and osmolarity. Each extender has a unique composition and must be appropriately used for different animal species (Alkan *et al.,* 2002). However, research on extenders specifically for bantam chicken semen remains limited. The commonly used semen extenders for avian species include Beltsville Poultry Semen Extender (BPSE), modified Sasaki extender, and Blumberger Hahnen Sperma Verdünner (BHSV) extender. The BHSV extender is advantageous due to the ease of its preparation and its effectiveness in preserving both chilled and frozen sperm (Kunkeaw *et al.,* 2021). A comparative study has shown that the simple medium for assisted reproductive technology (SMART) extender outperforms Tris-based and full cream milk extenders in prolonging the semen lifespan and activity in Lohman brown roosters (Tahseen *et al.,* 2019). Moreover, the incorporation of bovine serum albumin (BSA) in semen extenders improved sperm motility and velocity in turkeys (Bakst and Cecil, 1992).

Apart from the use of chilled semen, cryopreservation serves as an alternative to maintain breeding lines and conserve the genetic diversity of species or breeds (Woelders, 2021). This involves the use of cryoprotectants (CPAs) to protect the sperm from the detrimental effects of cryoinjury during the freeze-thaw cycles. While glycerol has been commonly used in the cryopreservation of chicken semen, it has been found to cause a contraceptive effect that adversely impacts fertility (Long and Kulkarni, 2004). Tarvis *et al.* (2013) examined the effect of dimethylacetamide (DMA), another cryoprotectant, on frozen-thawed chicken semen. Their results showed that 41% of the sperm were motile, and 45% had intact membranes following DMA treatment.

In this study, DMA was used as a cryoprotectant for the freezing process. Owing to the limited availability of human serum albumin (HSA) in the SMART extender, bovine serum albumin (BSA) was employed as a substitute in the modified extender. This investigation focuses on evaluating the effects of the modified SMART extender on sperm longevity and the quality of frozen-thawed semen relative to the BHSV extender.

Materials and Methods

Experimental animals: Approval for all experimental procedures in this study was obtained from the Institutional Animal Care and Use Committee (IACUC) of the Faculty of Veterinary Medicine, Chiang Mai University (Ref. No. S20/2564), in accordance with international standards. A total of 20 healthy male Bantam chickens (Gallus gallus), aged over 8 months and weighing between 0.7 and 0.8 kg, were used in the experiment. The chickens were obtained from the Department of Animal Science, Rajamangala University of Technology Lanna, Lampang. They were kept in individual high-raised cages (120×120×120 cm) and exposed to natural light for 12 hours a day. All chickens were fed 200 g of commercial poultry pellets containing 17% protein and provided with water ad libitum. Semen samples were collected once a week for the entire duration of 3 weeks.

Extender preparation: The modified SMART extender was prepared in a 50 mL centrifuge tube containing 29 mmol sodium bicarbonate, 3.2 g sodium lactate, 6.0 g sodium chloride, 0.4 g potassium chloride, 0.27 g calcium chloride, 0.01 g sodium pyruvate, 20% of bovine serum albumin (BSA) and phenol red in 1 liter of distilled water (Tahseen *et al.,* 2019). On the other hand, the BHSV extender was formulated in a 50 ml centrifuge tube, which consisted of 0.25 g glucose, 1.425 g sodium glutamate, 0.25 g potassium acetate, 0.035 g magnesium acetate, 0.125 g Myo-inositol and 50 ml of distilled water (Seigneurin *et al.,* 2013).

Experimental design: Semen samples were collected from the experimental animals weekly for 3 weeks. Only samples that met the criteria of sperm motility (≥70%) were included in the two experiments described below:

Experiment 1: For the longevity experiment, 20 µL semen from each sample was diluted with the modified SMART extender, and the semen's longevity was assessed after 1, 4, 24, and 48 hours.

Experiment 2: In the cryopreservation experiment, the remaining semen was divided equally and diluted with 6% and 9% dimethylacetamide (DMA) in both the modified SMART extender and BHSV.

Table 1 Experimental groups of Bantam chicken semen in this study.

Treatment Group	Extender and cryoprotectant combination
Group 1	Modified SMART extender diluted with 6% dimethylacetamide
Group 2	Modified SMART extender diluted with 9% dimethylacetamide
Group 3	BHSV extender diluted with 6% dimethylacetamide
Group 4	BHSV extender diluted with 9% dimethylacetamide

Semen collection: Semen was collected in a 1.5 ml sterile centrifuge tube using the dorso-abdominal massage method. After cleaning the cloacal area, each chicken was gently massaged at the back and stroked repeatedly close to its tail. This led to the raising of the tail and induced tumescence of the phallus. Semen was ejaculated following the application of slight pressure to the inverted cloaca (Malik *et al.,* 2013).

Fresh semen analysis: The color and volume of the semen were assessed macroscopically, followed by an evaluation of the semen's microscopic characteristics. Specifically, sperm morphology and viability were analyzed in 200 spermatozoa per sample by diluting the semen, smearing it on a glass slide, and staining it with eosin-nigrosin. Sperm concentration was determined using a hemocytometer and expressed as sperm/ml. For total motility assessment, a small aliquot of the semen sample was placed on a glass slide, covered with a coverslip, and observed under a light microscope.

Sperm longevity: Chicken semen with semen motility above 70% (Sonseeda *et al.,* 2013) was diluted with a modified SMART extender in the ratio 1:2 to 1:4 up to the desired concentration (2×10^8 sperm/mL). The diluted semen was stored at 5°C, and sperm longevity of individual semen was analyzed using total sperm motility and viability, which was observed under the microscope after 1 hour, 4 hours, 24 hours, and 48 hours of being stored in a modified SMART extender.

Semen cryopreservation: Bantam chicken semen was divided into 4 treatment groups, as shown in Table 1. Samples from each group were loaded into 1 ml cryotubes, equilibrated at 5°C for 15 minutes, frozen in liquid nitrogen vapor at 3 cm above the liquid nitrogen level for 20 minutes, and then stored at -196°C until analysis (Tarvis, 2013).

Semen analysis after thawing: Immediately after thawing, sperm motility, velocity, and kinematics were determined using a computer-assisted sperm analysis system (CASA).

Statistical analysis: The longevity and quality of frozen-thawed semen were presented as mean±standard error of the mean (SEM). Variance data were analyzed using the generalized estimating equation (GEE) in R Studio version 3.0.1 (2021), with a significance threshold set at $P<0.05$.

Result

Sperm longevity: Table 2 indicates that fresh Bantam chicken semen had the highest motility and viability when compared to chilled semen at 4-, 24-, and 48-hours post-collection ($P<0.05$). In particular, the mean motility of fresh semen was recorded at 73.37±2.03, and the mean viability was 77.42±3.30. The motility of modified SMART extender-supplemented chicken semen significantly declined to 67.21±2.42, 12.65±2.29, and 0.28±0.17 after 4, 24, and 48 hours, respectively ($P<0.05$). A similar trend was likewise observed in sperm viability, which decreased drastically over time from 69.47±2.55 at 4 hours to 32.89±2.88 at 24 hours and 16.96±1.74 at 48 hours post-collection ($P<0.05$).

Sperm Cryopreservation: In general, there is a decrease in sperm motility and viability after the freezing-thawing procedures. Table 3 presents the comparison of frozen semen parameters between the modified SMART extender and BHSV extender. Except for total motility, HAC, and STR, results demonstrated that the BHSV extender substantially improved sperm viability and motility parameters in Bantam chickens ($P<0.05$). Between the tested cryoprotectant levels, a 6% dimethylacetamide concentration significantly enhanced viability in both the modified SMART extender and the BHSV extender ($P<0.05$). In the modified SMART extender, notable improvements were observed in progressive motility, VCL, DCL, DAP, ALH, and BCF after using the same DMA concentration ($P<0.05$). Conversely, varying DMA levels did not significantly affect other CASA parameters in the BHSV extender ($P<0.05$).

Discussion

Conventional semen evaluation procedures in avian species include the assessment of various parameters such as color, volume, turbidity, motility, and viability. These characteristics have been proven to be related to the fertilizing potential of fresh spermatozoa (Getachew, 2016). High-quality sperm increases the likelihood of successful fertilization and embryo survival. Despite current efforts, the use of frozen semen in poultry remains below acceptable levels compared to their fresh counterparts due to the lack of standardized semen cryopreservation extenders suited for these species (Petričáková *et al.,* 2022). Hence, this present work focuses on the effects of the modified SMART extender on sperm longevity and frozen-thawed semen quality when combined with different MDA concentrations.

Apart from cryopreservation, chilling is another method of avian semen storage. The motility of fresh semen and chilled semen 4 hours post-collection in Bantam chickens in this study was comparable to the values obtained in Lohman brown chicken semen using the SMART extender (Tahseen *et al*., 2019). These findings may be attributed to the antioxidant properties of bovine serum albumin (BSA) and the incorporation of pyruvate as an energy source in the modified extender mixture (Tahseen *et al.,* 2019). In other animal species, BSA helps maintain sperm

morphology, coats the sperm plasma membrane against cold shock, and enhances the scavenging of oxidative stress-induced free radicals during the freeze-thaw cycles (Behnamifar *et al.*, 2021). Supplementation of BSA in the avian semen extender is also beneficial for sperm motility, velocity, and viability (Sarkar *et al.*, 2020). The motility results in the modified SMART extender saw a significant drop to 12.65±2.29 at 24 hours and 0.28±0.17 at 48 hours, implying that semen samples stored beyond 24 hours may no longer be viable for cryopreservation procedures. Therefore, evaluating sperm motility between 4 and 24 hours after collection is crucial to establish the maximum storage duration for avian semen.

A comparison of the effects of DMA concentrations on frozen-thawed semen quality demonstrated that 9% DMA yields better motility and viability than 6% DMA (Table 3). Mosca *et al.* (2019) found that DMA-cryopreserved chicken semen exhibited high viability and progressive motility, along with superior VCL, VAP, ALH, and BCF values. This current experiment also evaluated the quality of frozen-thawed semen between the modified SMART extender and the BHSV extender, suggesting that the latter results in better motility but has little effect on viability percentage. The values observed here are consistent with those reported by Kunkeaw *et al.* (2021) for Pradu Hang Dam chickens using the BHSV extender. According to Bakst and Cecil (1992), BSA can rapidly increase motility in the initial stage of the cryopreservation procedure, which, in turn, causes significant energy consumption during equilibration before freezing. Consequently, the remaining energy reserves after thawing are much lower, resulting in concomitantly lower motility.

In this study, we modified the formulation of the SMART extender from Tahseen *et al.* (2019) by substituting HSA with BSA. Differential scanning calorimetry (DSC) has revealed differences in fatty acid composition between bovine and human albumins, with human albumin being more thermally stable than bovine albumin (Michnik *et al.*, 2006). As a result, modifications to the albumin source might impact sperm motility due to these fatty acid differences (Dixon and Kreider, 1981; Bakst and Cecil, 1992). Hence, the distinct type of albumin used in each study may explain the varying outcomes in our study compared to Tahseen *et al.* (2019).

In conclusion, the modified SMART extender broadens the current extender options available for poultry semen preservation. It effectively maintains sperm longevity for up to 4 hours post-collection for short-term storage of Bantam chicken semen. However, it is less suitable for use in cryopreservation procedures than the BHSV extender. While this study centers on motility and viability as semen quality indicators for the modified SMART extender, future studies should investigate additional semen parameters and evaluate their long-term impact on fertility and hatchability outcomes.

Table 2 Effect of the modified SMART extender on Bantam chicken sperm parameters immediately after collection and at 4 h, 24 h, and 48 h post-collection.

Sperm trait	Fresh semen	Chilled semen		
		4 hours	24 hours	48 hours
%Motility	73.37±2.03^{a}	67.21±2.42^{b}	12.65±2.29^{c}	0.279±0.165^{d}
%Viability	77.42±3.30^{a}	69.47±2.55^{b}	32.89±2.88^{c}	16.96±1.74^{d}

^{a,b,c,d} Values within a row differ significantly at P<0.05 between treatments.

Table 3 Computer-assisted sperm analysis system (CASA)-derived frozen-thawed parameters of Bantam chicken semen subjected to BHSV extender and modified SMART extender at 6% and 9% DMA.

Sperm trait	BHSV extender		Modified SMART extender	
	6% DMA	9% DMA	6% DMA	9% DMA
Viability	35.1±1.377^{b}	46.61±1.71^{a}	29.36 ±2.57^{c}	38.26±1.39^{b}
Total motility	40.95±3.15^{a}	44.59±4.56^{a}	36.07±1.62^{a}	42.09±3.17^{a}
Progressive motility	21.56±2.10^{a}	25.54±5.42^{a}	13.47±1.00^{b}	16.83±1.46^{ab}
VCL	45.26±2.61^{ab}	49.32±5.55^{a}	34.62±1.47^{c}	39.25±1.34^{b}
VSL	20.30±0.92^{a}	24.16±2.34^{a}	16.57±1.09^{b}	16.58±0.54^{b}
VAP	24.63±1.11^{a}	28.83±2.97^{a}	19.52±1.08^{b}	20.15±0.61^{b}
DCL	9.69±0.74^{a}	9.58±0.96^{a}	7.32±0.28^{b}	8.25±0.38^{a}
DSL	2.55±0.16^{ab}	2.80±0.37^{a}	1.93±0.04^{b}	2.079±0.09^{b}
DAP	3.82±0.27^{a}	4.12±0.53^{a}	2.81±0.05^{c}	3.13±0.18^{b}
ALH	0.47±0.03^{a}	0.49±0.06^{a}	0.34±0.01^{b}	0.39±0.02^{a}
BCF	3.34±0.39^{a}	3.73±0.71^{a}	1.87±0.10^{b}	2.50±0.27^{a}
HAC	0.15±0.01^{a}	0.16±0.01^{a}	0.14±0.01^{a}	0.15±0.01^{a}
WOB	0.55±0.02^{ab}	0.59±0.01^{a}	0.56±0.02^{ab}	0.54±0.014^{b}
LIN	0.45±0.02^{ab}	0.49±0.02^{a}	0.48±0.03^{ab}	0.44±0.02^{b}
STR	0.83±0.14^{a}	0.84±0.01^{a}	0.85±0.01^{a}	0.82±0.01^{a}

^{a,b,c,d} Values within a row differ significantly at P<0.05 between treatments.

VCL, curvilinear velocity; VSL, straight-line velocity; VAP, average path velocity; DCL, distance curved line; DSL, distance straight line; DAP, distance average path; ALH, amplitude of lateral head displacement; BCF, beat cross frequency; HAC, head activity; WOB (VAP/VCL×100), wobble; LIN (VSL/VCL×100), linearity, and STR (VSL/VAP×100), straightness.

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