Kasetsart J. (Nat. Sci.) 43 : 269 - 279 (2009)

Effects of Extender, Cryoprotectant and Freezing Protocols on Post-thaw Sperm Motility, Morphology and Viability of Three-yellow Cocks (*Gallus domesticus*) Spermatozoa

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ABSTRACT

Semen samples collected from 10 three-yellow cocks (*Gallus domesticus*) were pooled and treated to determine the effect of extender and cryoprotectant (experiment 1) and the effect of freezing protocol and cryoprotectant (experiment 2) on frozen/thawed semen quality. In the first experiment, semen samples were diluted 1:1 (v:v) with two different semen extenders, Beltsville poultry semen extender (BPSE) or modified Tyrode's medium (TALP) and cryopreserved with either 6% dimethyl acetamide (DMA) or 8% dimethyl sulfoxide (DMSO). The best result ($p<0.05$) was obtained for TALP extender + 8% DMSO, with values of 57.32% for viability, 56.57% for normal morphology and 48.50% for motility. In experiment 2, two different freezing protocols (one-step and two-step) were evaluated. The results showed that there was no difference in post-thaw motility and viability of the spermatozoa (sperm) when either the one-step or the two-step protocol was applied with 8% DMSO. In contrast, the sperm motility and viability with the one-step protocol were better ($p<0.05$) than those with the two-step protocol when 6% DMA was used.

This study indicated that from *in vitro* analysis, TALP with 8% DMSO was the preferred freezing solution for three-yellow cock sperm and this could be carried out using a one-step freezing protocol.

Key words: three-yellow chicken, *Gallus domesticus*, extender, cryoprotectant, frozen semen

INTRODUCTION

The three-yellow chicken (*Gallus domesticus*), which has yellow plumage, yellow skin and yellow shanks, originated from China. Its appearance, meat flavor and meat texture are the main attributes that attract customers (Yang and Jiang, 2005). Recently, it has been used for crossbreeding to produce a kind of quality chicken in Thailand.

Artificial insemination (AI) with cooled storage semen in poultry is a valuable tool for lowering costs and increasing the usage of males, especially great grandparent and grandparent, in

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Received date : 17/12/08 Accepted date : 03/03/09

the breeder operation (Hammerstedt, 1992; Reddy, 1995; Blesbois and Brillard, 2007). However, AI with frozen/thawed poultry semen is not cost effective for commercial production (Donaghue and Wishart, 2000; Fulton, 2006; Blesbois and Brillard, 2007). Cryopreservation of poultry semen is the only effective method to provide more opportunities for the management of genetic resources (Fulton, 2006; Blesbois, 2007), particularly in a situation involving a pandemic outbreak of some infectious disease, such as avian influenza (Capua and Alexander, 2006; Morgan, 2006). Poultry semen diluted with various semen extenders and stored at 2-5°C for up to 48 h yielded no significant loss in fertilizing capacity (Etches, 1996). However, the fertility rates with frozen/thawed semen were considerably lower and highly variable (reviewed in Donaghue and Wishart, 2000; Blesbois, 2007). Since the requirements for processing the semen of various poultry species for cryopreservation are different, it is important to use a suitable cryoprotective medium, a correct time and temperature of equilibration, as well as the proper cooling and thawing rates. Different semen extenders, cryoprotectants, temperatures and times of equilibration and different rates of temperature decrease and increase during freezing and thawing have been tested (Chelmonska *et al*., 2006).

There are many semen extenders available for poultry semen, with both published recipes and commercially available products, with the basic characteristics common to nearly all extenders being to maintain pH, osmolarity and provide an energy source for sperm. Several investigators have compared the composition of various diluents and summarized fertility data across studies, but there is no standard diluent for poultry semen and the studies varied in experimental design (Donoghue and Wishart, 2000). Sexton (1988) compared the commercial diluents: Beltsville Poultry Semen Extender (BPSE), Instrument for Veterinary Medicine (IMV) and Minnesota Turkey Growers Association (MTGA) for holding turkey semen for 24 h at 5°C, with the results indicating that the sperm stored with BPSE had high motile, fertility rates and a low rate of lyses. However, Sontakke *et al*. (2004) indicated that modified Tyrode's medium (TALP) was the most suitable semen extender for blue rock pigeon (*Columba livia*) when compared with turkey sperm extender (TSE), chicken sperm extender (CSE), mouse sperm extender (CZB), Ham's F-10 and Tyrode's medium.

When freezing poultry semen, glycerol is as effective cryprotective for fowl sperm, but glycerol is a contraceptive for intravaginally inseminated chicken or turkey sperm (Donoghue and Wishart, 2000). It has been suggested that DMA and DMSO could be used as a freezing cryoprotectant to replace glycerol, with the aim of overcoming the contraceptive action and simplifying the insemination protocol by avoiding the need to remove the cryoprotectant before insemination (Tai *et al*., 2001). Most protocols for avian semen cryopreservation have been applied using a two-step protocol adapted from Blesbois and Brillard (2007) which involves cooling for 30 min and equilibrating for 30 min. A one-step cryopreservation protocol was adapted to reduce the time for preparation by adding semen extender and cryopreservative in a single dilutor.

This study aimed to investigate the effect of semen extenders (BPSE and TALP), cryoprotectants (6 % DMA and 8% DMSO) and the freezing protocol (one-step and two-step) on the post-thaw sperm motility, morphology and viability in the three-yellow chicken. The results will help to develop the further implementation of AI with frozen/thawed semen in the three-yellow chicken.

MATERIALS AND METHODS

Animals and semen collection

Ten three-yellow cocks, aged 1-3 years old were kept in individual pens and housed under

appropriate management conditions (ambient temperature about 25°C, natural light and *ad libitum* feed and water). Semen was collected twice a week during May to July 2007, using a massage collection technique (Burrows and Quinn, 1937). Special care was taken during semen collection, with any samples contaminated with feces being discarded. The semen collection from 10 cocks was completed within 20-25 min, pooled and evaluated as one sample.

Semen evaluation

In the freshly collected semen, the following parameters were examined: volume, pH, osmolarity, sperm concentration, percentages of live, morphologically normal and motile sperm, as well as sperm velocity parameters. Sperm concentration was determined using a hemacytometer. Sperm viability and morphology were evaluated in an eosin-nigrosin smear under a light microscope. A single observer analyzed all preparations.

In addition, the semen samples were evaluated for motility and velocity parameters by Computer Assisted Semen Analysis (CASA; Hamilton Thorne Research. Beverly, MA, USA). The parameters evaluated by CASA were: total motile sperm; percentage of progressive motile sperm; average path velocity (VAP; μm/s); progressive velocity (VSL; μm/s); curvilinear velocity (VCL; μm/s); beat cross frequency (BCF; Hz); amplitude of lateral head displacement (ALH; μm) and linearity of track (LIN; %) The instrument setting was adapted from Sontakke *et al*. (2004)

Experiment 1 - The effect of extenders and cryoprotectants on frozen/thawed semen quality

The pooled semen was divided into two equal parts, which were diluted in a two-step process according to Blesbois and Brillard (2007). Briefly, each part (1 ml) of the semen sample was diluted 1:1 (v:v) at 37°C with either BPSE or TALP extender and cooled down to 5°C for 30 min. Each extender was further made up into two parts by diluting with the same extender to a final dilution of 1:2 (v:v; semen:extender) at 5°C with the same extenders containing DMA or DMSO to a final dilution of either 6% or 8% respectively. After equilibration at 5°C for 30 min, the semen was loaded into a 0.50 ml straw (I.M.V., L'Aigle, France) and then placed 5 cm above liquid nitrogen vapor for 10 min, before being plunged into liquid nitrogen. The osmolarity of the extender with and without cryoprotectant was measured by an osmolarity meter (Osmomat 030, Geprufte Sicherheit, Berlin, Germany). The values are shown in Table 1.

After one week in storage, the semen samples were thawed by placing the straws into a water bath (40°C) for 10 sec and the post-thaw semen quality was evaluated. Sperm longevity was determined by diluted 1:5 (v:v) semen with the same extenders that had been used for freezing. The diluted semen aliquots were maintained at 37°C in an incubator (5% CO_2 in air) and the percentage of motile sperm was determined using CASA every hour for 6 h. The entire experiment was replicated four times.

Table 1 Osmolarity of semen extenders with and without cryoprotectant.

Cryoprotectant	Osmolarity (mOsm/kg)	
	BPSE	SP-TALP
Without cryoprotectant	330	344
With 6% DMA	1,240	1,727
With 8% DMSO	1,178	2,577

Experiment 2 - Effects of freezing protocols and cryoprotectants on frozen/thawed semen quality

Each pooled semen sample was divided into four parts and diluted with TALP as a freezing extender, chosen based on the results of experiment 1. The semen samples were subjected to two different freezing protocols, one-step and two-step. In the one-step protocol, each sample of semen was diluted 1:2 (v:v) with TALP and cryopreserved with either 6% DMA or 8% DMSO, whereas in the two-step protocol, each sample of semen was diluted 1:1 (v:v), cooled to 5°C for 30 min and diluted again in the same extender added previously (either 6% DMA or 8% DMSO). After the semen samples had been equilibrated at 5°C for 30 min, each sample was loaded into a 0.50 ml straw (I.M.V., L'Aigle, France) and placed 5 cm above liquid nitrogen vapor for 10 min, before being plunged into liquid nitrogen.

Statistical analysis

Statistical analysis of the data from the four replicates was carried out using ANOVA and Duncan's multiple range test (SAS version 6.12. SAS Institute Inc., Cary, NC, USA). Differences were considered significant at $P < 0.05$. Results were determined as means ± standard error of mean (SEM) of the four replicates.

RESULTS

Fresh semen (n=10) was white to cream in color. The volume of semen obtained was in the range 1.70-2.80 ml. Characteristics of the samples are presented in Table 2. The semen pH was slightly basic and the average osmotic pressure varied from 328 to 402 mOsmol/kg.

In the first experiment, the proportion of morphologically normal and viable sperm were higher in samples treated with TALP than in those treated with BPSE, regardless of whether 6% DMA or 8% DMSO was used (Table 3). All values were significantly lower than those observed before the freezing process (92% normal and 83% viable sperm). For all TALP treatments, the sperm frozen and thawed in straws with 8% DMSO had normal morphology and viability (57 and 57%, respectively) and these values were significantly greater than those with 6% DMA (50 and 50%, respectively).

The total motility before semen dilution did not differ between groups. However, the total motility mean immediately after semen dilution,

Table 2 Characteristics of fresh, pooled semen from ten three-yellow cocks.

Parameter	Mean±SEM	Range
Pooled volume (ml)	2.22 ± 0.04	1.70 - 2.80
pH	7.82 ± 0.04	7.44 - 8.22
Osmolarity (mOsm kg^{-1})	347.75 ± 3.39	328.00 - 402.00
Sperm concentration (× 10^6 ml^{-1})	3,463.75 ± 16.34	3,210.00 - 3,635.00
Motile sperm (%)	89.17 ± 0.67	80.00 - 95.00
Live sperm (%)	92.38 ± 0.93	88.00 - 94.00
Morphological normal sperm (%)	82.82 ± 0.66	80.00 - 85.00
Morphological abnormal sperm		
Macrocephalic	2.16 ± 0.18	1.50-3.50
Amorphous head	1.42 ± 0.13	1.00-2.50
Bent mid piece	1.53 ± 0.10	0.50-2.50
Bent tail	1.17 ± 0.21	0.50-2.75
Cytoplasmic droplet	2.25 ± 0.12	1.50-3.00

equilibration and post thawing did differ significantly between groups. (Figure 1a). The percentage of progressive motile sperm after semen dilution and cooling did not differ between extenders, but did differ significantly between extenders in the semen after equilibration and post thawing (Figure 1b).

Regarding the velocity parameters, VAP, VSL, VCL, ALH and LIN did not differ in the diluted semen immediately or after cooling. VAP and LIN did not differ between groups of extenders and cryoprotectants in post-thaw semen. However, the post-thaw semen treated with BPSE and 8% DMSO had more VSL, VCL and ALH than the other group (Figures 1c – 1h).

The *in vitro* sperm longevity analysis of semen diluted with TALP and BPSE (1:5, v:v) without cryoprotectants and cryopreservation (control group) is presented in Table 4. The percentage of motile sperm decreased from 83.00 to 58.00% and 83.00 to 46.25 % after 6 h *in vitro* for TALP and BPSE, respectively, which was a significant decrease ($p<0.05$) from 1 h onwards. After 4 h of incubation, a significantly higher percentage of motile sperm ($p<0.05$) was observed in TALP than in BPSE. For the post-thaw sperm, the sample treated with TALP had a significantly higher percentage of motile sperm ($p<0.05$) than for BPSE. The TALP group treated with 8% DMSO had a higher percentage of motile sperm (10%) and no motile sperm at 6 h, whereas in BPSE group, no motile sperm were present at 4 h.

In the second experiment, the semen samples treated with 8% DMSO by freezing with either the one-step and two-step protocol did not differ with regard to the proportion of morphologically normal sperm or viability (50 and 51%; 55 and 56%, respectively). However, semen samples treated with 6% DMA by freezing with the one-step protocol had a significantly higher ($p<0.05$) proportion of morphologically normal sperm and viability than by freezing with the two-step protocol (49 and 40%; 51 and 48%). The one-step and two-step protocols with 8% DMSO both had a significantly higher ($p<0.05$) proportion of morphologically normal sperm and viability than from freezing with 6% DMA (Table 5).

In terms of the sperm motility and velocity parameters, semen dilution immediately produced significant differences in total motile, percentage of progressive motile, VAP, VSL, VCL, ALH and LIN. The one-step freezing groups had lower values than the two-step freezing groups, regardless of whether either 6% DMA or 8% DMSO had been used. The post-thaw semen samples did differ in the percentage of progressive motile sperm, VAP, VSL, VCL, ALH, BCF and LIN (Figures 2a – 2h).

Table 3 Effect of extender and cryoprotectant on post-thaw sperm viability and morphology of the three- yellow cock.

Sperm parameter (%)	TALP		BPSE	
	8% DMSO	6% DMA	8% DMSO	6% DMA
Live sperm	57.32±0.20^{a}	50.50±0.13^{b}	46.58±0.17^{c}	45.08±0.21^{c}
Morphological normal	56.67±0.55^{a}	49.25±0.74^{b}	42.33±0.79^{c}	40.75±0.34^{c}
Morphological abnormal				
Macrocephalic	2.33±0.22^{a}	4.33±0.21^{b}	4.33±0.22^{b}	4.58±0.12^{b}
Amorphous head	1.92±0.21^{a}	3.25±0.22^{b}	2.42±0.13^{ab}	2.42±0.13^{ab}
Bent mid piece	2.42±0.15^{a}	4.83±0.16^{b}	4.08±0.26^{b}	4.83±0.24^{b}
Bent tail	17.92±0.39^{a}	18.58±0.40^{ab}	20.92±0.22^{c}	20.17±0.24^{bc}
Cytoplasmic droplet	2.08±0.16^{a}	1.25±0.12^{ab}	2.50±0.16^{ac}	2.33±0.12^{ac}

Values represent mean± standard error of mean (SEM)

^{abc} Values in the same row that do not share a common superscript differ significantly ($p<0.05$)

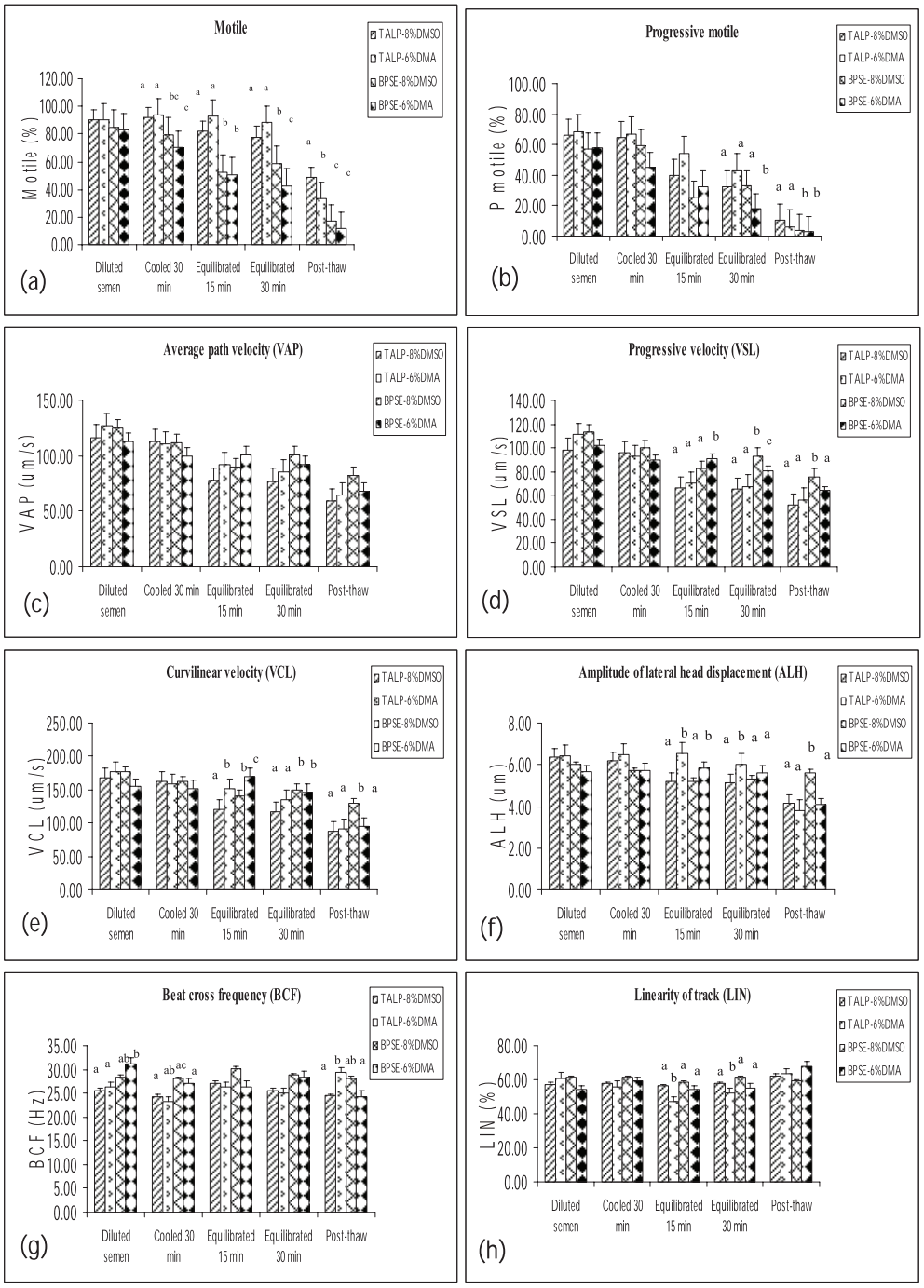

Figure 1 Effect of extender and cryoprotectant on three- yellow cock sperm motility and velocity parameters during the freezing process (a) motile sperm, (b) progressive motile sperm, (c) average path velocity, (d) progressive velocity, (e) curvilinear velocity, (f) amplitude of lateral head displacement, (g) beat cross frequency, and (h) linearity of track. The different superscript letters (^{a,b,c}) over the bars indicate significant differences within groups.

Table 4 Effect of extender and cryoprotectant on post-thaw percentages of motile sperm of the three-yellow cock incubated at 37°C for 6 h.

Group	0 min	30 min	1 h	2 h	3 h	4 h	5 h	6 h
Fresh								
TALP	83.00 ±0.41^{A,a}	83.25±0.48^{A,a}	80.75±0.48^{A,b}	79.75±0.63^{A,bc}	79.00±0.41^{A,c}	78.50±0.64^{A,c}	70.75±0.63^{A,d}	58.00 ±0.58^{A,e}
BPSE	83.00±0.41^{A,a}	83.00±0.41^{A,a}	80.50±0.29^{A,b}	79.25±0.48^{A,b}	77.75±0.48 ^{A,c}	66.50±0.64^{B,d}	56.75±0.48^{B,e}	46.25±0.63^{B,f}
Frozen-thawed								
TALP + 8% DMSO	51.50±2.66^{B,a}	47.50±0.95 ^{B,a}	31.50±1.32^{B,b}	27.75±0.75^{B,b}	22.25±1.79^{B,c}	16.50±2.18^{C,d}	10.25±1.10^{C,e}	0 ^{C,f}
TALP + 6% DMA	34.50±6.94^{C,a}	30.25±6.98^{C,ab}	22.75±2.81^{C,bc}	17.50±2.50^{C,c}	11.75±1.49^{C,d}	0^{D,e}	0^{D,e}	0^{C,e}
BPSE + 8% DMSO	20.75±1.87^{D,a}	18.75±1.87^{D,a}	13.50 ±0.95^{D,b}	7.25±0.48 ^{D,c}	2.75±0.94 ^{D,d}	0^{D,d}	0^{D,d}	0^{C,e}
BPSE + 6% DMA	16.00±1.82^{D,a}	15.00±1.68^{D,a}	10.25±0.85^{D,b}	6.25±0.48^{D,c}	2.00±1.15^{D,d}	0^{D,d}	0^{D,d}	0^{C,d}

Value represents mean± SEM

^{ABCD} Values in the same column that do not share a common superscript differ significantly ($p<0.05$)

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For the *in vitro* sperm longevity analysis, the semen treated with 8% DMSO and freezing with the one-step and two-step protocols had high sperm motility and yielded motile sperm up to 5 h. In contrast, semen treated with 6% DMA and freezing with the one-step and two-step protocols had low sperm motility and only gave motile sperm up to 3 h of incubation *in vitro* (Table 6).

DISCUSSION

The study indicated that using TALP with 8% DMSO produced higher percentages of post-thaw live, morphological normal, motile and progressive motile sperm, as well as longer sperm longevity. In addition, the one-step freezing protocol yielded similar results to the two-step freezing protocol with this treatment.

The average semen volume, sperm concentration, pH and percentage of live and motile sperm for the three-yellow cocks used in this study were within the ranges reported for cocks of this species (Etches, 1996; Garner and Hafez, 2000). The osmotic pressure of fresh semen (328 – 402 mOsmol/kg) was also comparable to a previous study (320 – 340 mOsmol/kg; Lukaszewicz, 2002; cited by Chelmonska *et al.*, 2006).

As demonstrated by Sontakke *et al.* (2004), TALP supported the motility of pigeon sperm better than chicken-sperm extender, in which probably the albumin, lactate and pyruvate in TALP influenced sperm motility. Bovine serum albumin significantly increased the percentage of motile sperm in turkeys (Bakst and Cecil, 1992). Lactate and pyruvate were also components in cock seminal plasma (Etches, 1996). The present study confirmed the superior results of TALP compared to BPSE on sperm motility during incubation (Table 4) for both semen dilution and for freezing with 8% DMSO. Therefore, TALP provided more optimal conditions for storage of three-yellow cock sperm than BPSE.

Table 5 Effect of freezing protocol and cryoprotectant on post-thaw sperm viability and morphology of the three-yellow cock.

Sperm parameter (%)	One-step method		Two-step method	
	8% DMSO	6% DMA	8% DMSO	6% DMA
Live sperm	55.43±0.06[a]	51.08±0.14[b]	56.27±0.12 [a]	48.87±0.29[c]
Morphological normal	50.17±0.81[a]	48.83±0.63[b]	51.50±0.42[a]	40.42±0.20[c]
Morphological abnormal				
Macrocephalic head	3.33±0.20[a]	4.17±0.16[ab]	3.58±0.15[a]	4.58±0.13[b]
Amorphous head	1.92±0.22[a]	3.33±0.20[b]	1.92±0.22[a]	3.00±0.16[ab]
Bent midpiece	2.42±0.15[a]	4.83±0.16[b]	2.42±0.15[a]	4.67±0.16[b]
Bent tail	18.08±0.25[a]	18.67±0.39[ab]	16.50±0.14[a]	19.92±0.23[b]
Cytoplasmic droplet	2.08±0.16[a]	1.25±0.12[b]	2.25±0.19[a]	2.42±0.15[a]

Value represents mean± SEM

[abc] Values in the same row that do not share a common superscript differ significantly ($p<0.05$)

In combination with TALP, 8% DMSO protected sperm better than 6% DMA (Table 3, Figure 3a), but with no significant difference when BPSE was used. This may have been caused by the different interactions of DMSO and DMA with the components in TALP and BPSE. The type of extender was shown to have an effect on the degree of cytotoxicity of DMSO, by changing the suspension medium. These changes in the extender affected the cells and were limited to use with a phosphate buffer as a suspension (Sexton, 1975; 1980). The same circumstances could have occurred for DMA. Nevertheless, TALP with 8% DMSO was observed to be the preferred freezing solution for the three-yellow cock sperm compared to other combinations. The percentages of post-thaw live (57.32±0.40) morphological normal (50.17±0.63), motile (48.50±3.40) and progressive motile sperm (10.50±3.40) were considerably higher than in previous studies for: live and normal sperm; 21 – 39%; BPSE + 4.5% DMSO; (Van Voorst and Leenstra, 1995); and for live and normal sperm; 48.39±8.79%; motile sperm: 42.36±7.39%; progressive motile sperm: 7.48±2.85%; Lake diluent + 11% glycerol (Blesbois *et al.*, 2008).

In this study, the percentages of live and motile sperm as well as the sperm velocity were affected by cryopreservation, in agreement with a previous study (Blesbois *et al.*, 2008). The percentage of morphological abnormal sperm, especially bent tail, also increased significantly. This abnormality could have arisen from the physiological characteristics of avian sperm, which has quite a long tail. Donoghue and Wishart (2000) have suggested that its long tail (90 – 100 μm), compared to that of the bull sperm (50 μm), in addition to the cylindrical head shape, allowed for less cytoplasmic volume and therefore less ability to move cryoprotectants inside the sperm head, thus making it more susceptible to freezing damage.

The one-step freezing protocol with 6% DMA or 8% DMSO in this study resulted in a post-thawed semen velocity similar to that from the two-step freezing protocol with 6% DMA or 8% DMSO. The process of sperm cryopreservation imparted on sperm cells the stress of low-temperature and drastic osmotic change, resulting in cell damage (Meyers, 2005). The stress induced by the one-step freezing protocol due to osmotic pressure (Table 1) was initiated after dilution by decreasing the percentages of the progressive motile sperm and velocity parameters (Figures 4b – 4f). The deleterious effect of the cryoprotectant was not dependent on the concentration, although

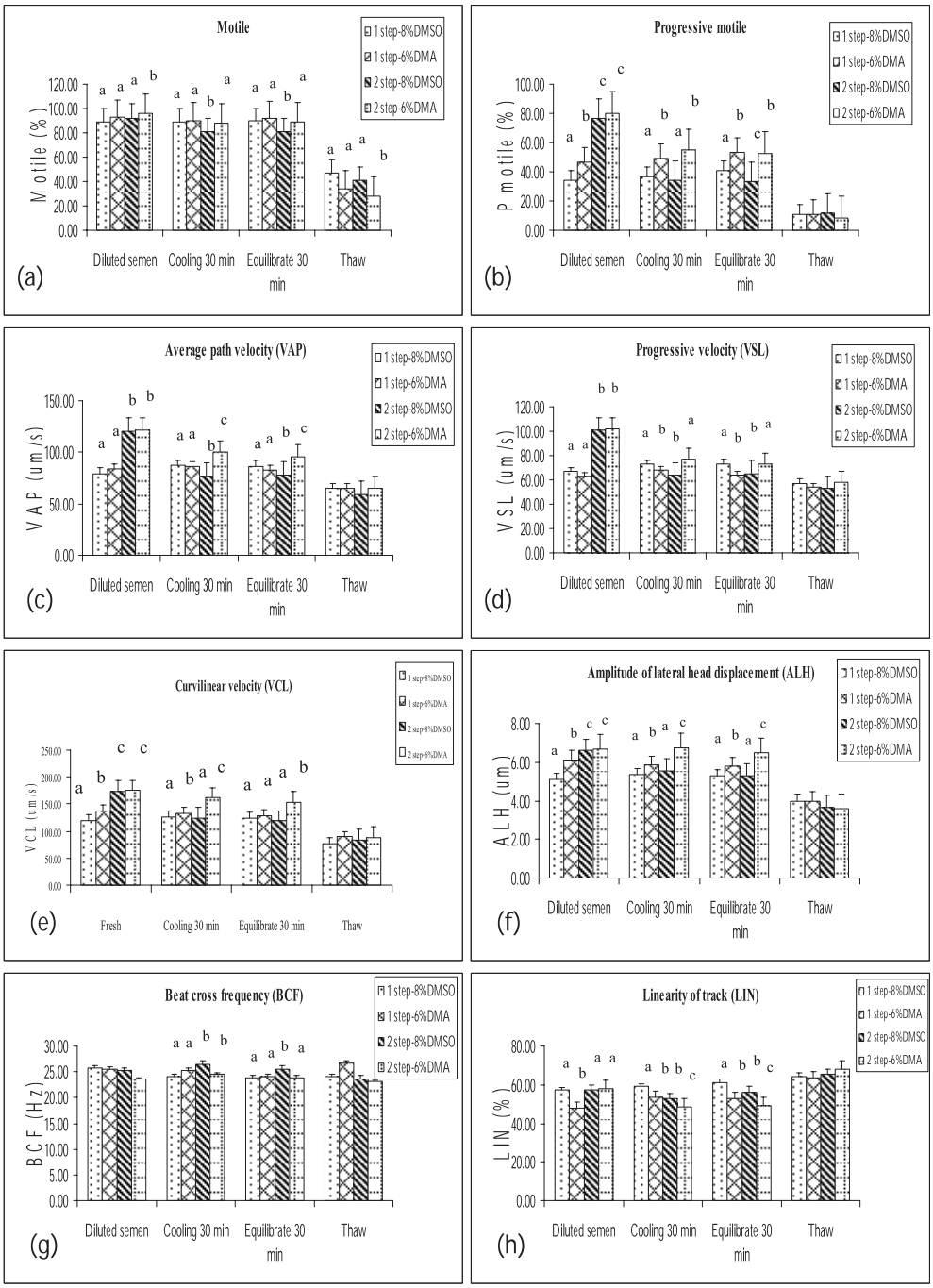

Figure 2 Effect of freezing protocol and cryoprotectant on three- yellow cock sperm motility and velocity parameters during the freezing process (a) motile sperm, (b) progressive motile sperm, (c) average path velocity, (d) progressive velocity, (e) curvilinear velocity, (f) amplitude of lateral head displacement, (g) beat cross frequency, and (h) linearity of track. The different superscript letter (^{a,b,c}) over bars indicate significant differences within groups.

Table 6 Effect of freezing protocol and cryoprotectant on post-thaw percentages of motile sperm of the three-yellow cock incubated at 37°C for 6 h.

Group	0 min	30 min	1 h	2 h	3 h	4 h	5 h	6 h
One-step + 8% DMSO	$46.50\pm0.96^{A,a}$	$44.00\pm1.41^{A,a}$	$31.75\pm0.34^{A,b}$	$26.75\pm0.75^{A,b}$	$20.25\pm1.89^{A,c}$	$14.50\pm2.22^{A,d}$	$9.25\pm1.31^{A,e}$	0^{f}
One-step + 6% DMA	$30.25\pm4.32^{B,a}$	$16.25\pm1.31^{B,b}$	$11.00\pm0.70^{B,c}$	$7.00\pm0.70^{B,cd}$	$2.50\pm1.91^{B,de}$	$0^{B,e}$	$0^{B,e}$	0^{e}
Two-step + 8% DMSO	$46.00\pm3.24^{A,a}$	$42.00\pm1.82^{A,a}$	$32.75\pm1.65^{A,b}$	$28.75\pm1.49^{A,b}$	$20.25\pm1.74^{A,c}$	$14.00\pm1.35^{A,d}$	$7.25\pm0.94^{A,e}$	0^{f}
Two-step + 6% DMA	$25.75\pm1.31^{B,a}$	$16.50\pm0.86^{B,b}$	$9.25\pm1.10^{B,c}$	$4.75\pm0.63^{B,d}$	$1.25\pm0.75^{B,e}$	$0^{B,e}$	$0^{B,e}$	0^{e}

Value represents mean± SEM

^{AB} Means in the same column that do not share a common superscript differ significantly ($p<0.05$)

^{abcdef} Means in the same row that do not share a common superscript differ significantly ($p<0.05$)

the osmotic pressure of the media increased regularly with the concentration. The hypertonicity of the media and the osmotic effect originating from the entry of the cryoprotectant into the cells are not the only factors responsible for the deleterious effect of the cryoprotectants (Tselutin *et al*., 1999). However, this deleterious effect on motile velocity may be minor or reversible, compared to the damage that can occur during freezing, therefore resulting in similar post-thaw semen quality to that under the two-step freezing protocol. The advantage of the one-step freezing protocol was that it was easier and took less time.

CONCLUSION

From the *in vitro* analysis, TALP with 8% DMSO was the preferred freezing solution for three-yellow cock sperm and could be accomplished with a one-step freezing protocol. Further implementation of AI with frozen/thawed semen using this method needs to be determined.

ACKNOWEDGEMENT

This research was supported by the Center of Agricultural Biotechnology through funding from Subject Graduate Study and Research in Agricultural Biotechnology under the Higher Education Development Project, Commission on Higher Education, the Ministry of Education.

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