CRYOPRESERVATION OF PHEASANT SEMEN: EFFECT OF DILUTION RATIO AND COOLING TIME ON SPERMATOZOA VIABILITY AND MOBILITY

M. Marzoni[1], A. Castillo[1], R. Chiarini[2], P.G. Bolognesi[3], and I. Romboli[1]

[1]Department of Animal Production, Pisa University, PISA, ITALY
[2]External collaborator
[3]Department SPVPA, Bologna University, BOLOGNA, ITALY
ghita@vet.unipi.it (e-mail of the corresponding author, M. Marzoni)

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ABSTRACT

Aim of the present study was to investigate the cryopreservation of pheasant semen by adopting the freezing/thawing protocol by Tselutin et al. (1999) with some modifications. Semen was collected by abdominal massage twice a week. Evaluations were performed on pooled semen from fifteen males (*Phasianus colchicus mongolicus*). Two semen dilutions (DIL) with Lake diluent (1:2 and 1:3, v/v; Lake, 1968) and two equilibration times (ET) at 5°C (10min and 30min) before dimethylacetamide (DMA) addition, were tested. Assessment of sperm mobility was performed by Accudenz methodology according to Froman's procedure (1997) and viability by eosin/nigrosin staining.
As expected, viability and mobility were strongly affected by the freezing/thawing procedure. ET did not affect mobility while influenced live sperm percentage during the DMA equilibration (DMA-Eq). Semen/diluent ratio significantly ($p<0.001$) modified the mobility parameter and the highest progressive movements of spermatozoa were obtained with the most diluted semen in each critical step of the cryopreservation procedure.
In conclusion, for pheasant semen cryopreservation, the 1:3 dilution ratio can be considered appropriate and the cooling time up to 30 minutes not crucial for the spermatozoa mobility and viability. Nevertheless, the deleterious effect of freezing/thawing procedure reduced live thawed spermatozoa to 24% and forward motility to 89% of the initial movement capacity.

INTRODUCTION

Artificial Insemination is a fundamental component of reproduction in few domestic avian species, like turkeys, where the difference in size of toms comparing to females makes natural mating difficult. At present, reduction in genetic variability of domestic livestock and increasing risk of line extinction due to health and safety reasons makes AI technique and semen preservation in domestic birds essential. Nevertheless, AI is based on the use of fresh semen, thus the conservation of live birds is necessary. Compared to mammalian species, in fact, birds have some unique physiological characteristics which might influence the use of stored semen, thus cryopreservation results difficult. According to Donoghue and Wishart (2000), one of the reasons avian spermatozoa do not survive the freezing process well is the limited cryoprotectant penetration into the sperm cell due to the small cytoplasmic volume of spermatozoa.
In addition, freezing procedures are not standardized for various species, therefore no appropriate methods of cryopreservation are now currently available for the poultry industry.
Recent studies on major domestic birds confirmed a species-specific ability of spermatozoa to resist various stresses caused by cryopreservation (Blesbois et al., 2005). Further research showed the need to set up semen freezing/thawing protocols, not only specifically on chickens, turkeys and guinea-fowls but on breeds/strains within the different species (Fulton, 2006; Long 2006).
Latest avian epidemic diseases showed the importance to protect genetic resources from pathological risk as well as the need to maintain flock of elite commercial stock at multiple locations, providing insurance against disease outbreaks and quarantine restrictions.
The cryopreservation technology has been studied for years, mainly in the chicken, but the results always showed a great reduction of the fertilised ability of the frozen/thawed sperm. Nevertheless, cryopreservation procedures based on the production of semen pellets by direct dropping of the sample

into liquid nitrogen give fertility rate of chicken semen similar to results obtained with fresh semen (Tselutin et al., 1999; Chalah et al., 1999; Woelders et al., 2006). Such good results obtained, open new positive perspectives for studies on cryopreservation of avian semen. To improve the knowledge on the use of this reproductive technique in domestic birds, the pellet freezing/thawing procedure using DMA as the cryoprotectant has been applied to pheasant, the most widespread game bird species, which has been recently undergone intensive rearing systems.
Aim of this study is to quantify the sperm damage in pheasants using the cryopreservation procedure for chickens (Tselutin et al., 1999), testing the effect of semen dilution and the 5°C equilibration time, verifying spermatozoa survival and *in vitro* fertilising capacity.

MATERIALS AND METHODS

Semen was routinely collected by dorso-abdominal massage twice a week from male pheasants (*Phasianus colchicus mongolicus*), breed under open air conditions. All ejaculates were maintained in a portable refrigerator at 18°C during collection. Thereafter, ejaculates were pooled and split into two samples, one diluted 1:2 and the other one 1:3 (v/v) in Lake diluent (Lake, 1968). Twelve 300µl aliquots of diluted semen (six per DIL) were rapidly cooled to 5°C and maintained at this temperature for two equilibration times (ET): 10 or 30 minutes (three aliquots per DIL for each ET). Rapid cooling was performed using a refrigerator preadjusted to -6±0.5°C and an immersion probe (BRE Elettronica Pisa, Italy) inside an additional sample was used to read the exact temperature. Afterwards, the following procedure was performed: semen aliquots were added with the DMA cryoprotectant at a final concentration of 6%, mixed manually for 1 minute and equilibrated for 4 minutes at 5°C and then dropped directly into a liquid nitrogen bath; volume of droplets was approximately 50µl. Pellets were stored in a liquid nitrogen tank. For the thawing procedure a thermoregulated hotplate at 60°C was used. So each pellet was put on the heated plate and pushed gently by means of a micropipette tip to recover the melted semen immediately. Sperm quality was assessed on fresh 1:1 diluted semen (FRESH) and on one aliquot of semen per DIL after every critical step of the procedure: 5°C equilibrated semen (COOLED), DMA equilibrated semen (DMA-Eq) and thawed semen (THAWED). The following sperm quality parameters were measured: viable spermatozoa (nigrosin/eosin staining) and sperm mobility by Accudenz® procedure (Froman, 1997). The experimental protocol was repeated four times on different days to increase the number of replicates within each treatment. Absorbance data, measured by a spectrophotometer (λ =550 nm), were transformed as a percentage of mobility of fresh diluted semen and results expressed as mobility loss. Percentage data were arcsine transformed prior to analysis. All data were tested for normality of distribution (Shapiro-Wilks test for normality) before performing the specific statistical analysis. Analysis of variance was used to process data on viability, absorbance and sperm mobility loss within each step of cryopreservation procedure; main factors were day of collection, dilution ratio, cooling time and procedure. All statistical data were conducted using JUMP (2002) software.

RESULTS

During this study fifteen ejaculates per day were collected. The mean volume of ejaculate was 121±12µl with a concentration of $8.2x10^9$/ml, a pH of 7.86±0,10 and a viability of 87±3.9% (mean±s.d.).
Percentage results of viability and mobility during the freezing/thawing procedure using two dilution rates and two interval times of equilibration at 5°C are presented in table 1. ET influenced live sperm percentage of DMA-Eq semen, resulting in higher viable cells with a 30-minute at 5°C before cryoprotectant addition. On the contrary, absorbance and mobility loss were not affected by ET. Semen/diluent ratio significantly ($p<0.001$) modified the mobility parameter and the highest progressive movements of spermatozoa were obtained with the most diluted semen in each critical step of the cryopreservation procedure. Consequently, considering the mobility expressed as loss in capacity of forward progressive movements, we obtained a 2% and 12% loss after the cooling step, a 23% and 30% loss after equilibration with DMA and a 88% and 90% loss after thawing for DIL-1:3 and DIL-1:2 semen, respectively.

After cooling and equilibration at 5°C for 10 minutes, viability resulted higher with DIL-1:2 and lower with DIL-1:3. After DMA addition/equilibration and after thawing, viability was similar between the four groups. Slightly better results on the viability of frozen-thawed spermatozoa (26%) were obtained when DIL-1:3 semen was equilibrated for 30 minutes at 5°C. Similar mean values of absorbance were obtained within each step of the cryopreservation procedure between groups. Nevertheless when considering the whole procedure slightly better forward movement capacity and, consequently, less mobility loss, were observed with DIL-1:3 equilibrated 10min.

Table 1 - Effect of dilution rate (DIL) and equilibration time at 5°C (ET) on quality parameters of semen after each critical step of the freezing/thawing procedure (mean ± S.E.)

		DIL (sperm:Lake diluent, v/v)					
		1:2		1:3			
		ET		ET		p-value	
Semen	n	10 min	30 min	10 min	30 min	DIL	ET
Viability %							
COOLED	16	86.5 ± 1.53a	83.5 ± 1.49ab	82.3 ± 1.08b	85.3±1.28ab	ns	ns
DMA-Eq	12	78.2 ± 1.75	81.0 ± 1.21	75.8 ± 1.09	80.1 ± 1.93	ns	*
THAWED	12	24.3 ± 1.70	24.1 ± 1.42	24.7 ± 1.96	25.8 ± 0.88	ns	ns
$Absorbance_{550}$							
COOLED	8	0.295±0.020	0.286±0.020	0.324±0.030	0.316±0.020	**	ns
DMA-Eq	6	0.216±0.005	0.220±0.013	0.241±0.013	0.250±0.010	*	ns
THAWED	24	0.031±0.002	0.035±0.001	0.041±0.003	0.039±0.002	**	ns
Mobility loss (% on FRESH mob.)							
COOLED		10.6	14.0	0.9	3.6	**	ns
DMA-Eq		31.9	29.5	24.8	21.3	*	ns
THAWED		90.4	89.7	87.7	87.9	**	ns

a,b,*: $p<0.05$; **:$p<0.01$

As expected, viability and mobility were strongly affected by the freezing/thawing procedure. This deleterious effect on both qualitative parameters of spermatozoa is evident in table 2.

Tab 2 – Changes of sperm viability and mobility during the cryopreservation procedure (mean ± S.E.)

	Semen			
	Fresh	Cooled	DMA-Eq	Thawed
Viability (%)	85.8 ± 1.04 A	84.4 ± 0.98 A	78.8 ± 0.80 B	23.9 ± 0.75 C
$Absorbance_{550}$	0.327 ± 0.015 A	0.305 ± 0.012 A	0.227 ± 0.006 B	0.037 ± 0.001 C
Mobility loss (% on fresh sperm mobility)	---	5.7 C	29.6 B	88.9 A

A,B,C: $p<0.001$

DISCUSSION

Initial viability and mobility of the ring-neck pheasant spermatozoa are very similar to data obtained in our previous study on this species (Marzoni et al., 2003).

When semen was processed for freezing, mobility and percentage of live spermatozoa decreased as critical steps of the cryopreservation procedure were passed through. Unexpectedly, the cooling treatment up to 30 minutes did not roughly affected spermatozoa, being sperm mobility and viability similar to fresh semen, in fact, the loss of mobility was less than 6% and dead spermatozoa increased about 1.5%. After subsequent critical steps, spermatozoa parameters changed significantly: the percentage of live cells reduced to 79 after cryoprotectant addition/equilibration and to 24 after

thawing. Regarding mobility, DMA equilibrated sperm and thawed sperm showed also a reduction: 30% and 89% on fresh semen, respectively.
The spermatozoa forward progression, representing an essential typology of movement of male reproductive cells, is a very useful indicator of sperm fertilizing capacity. In conclusion, dilution resulted to be an important factor influencing spermatozoa mobility and, for cryopreservation of pheasant semen, the 1:3 dilution ratio can be considered appropriate and the cooling time up to 30 minutes not crucial for the mobility and viability of spermatozoa. Nevertheless, further studies are necessary to improve the efficacy of semen cryopreservation and to set up a specific freezing/thawing protocol for pheasant.

REFERENCES:

Blesbois, E., Grasseau, I. and Seigneurin, F. (2005) *Reproduction,* **129,** 371-378.
Chalah, T., Seigneurin, F., Blesbois, E. and Brillard, J. P. (1999) *Cryobiology,* **39,** 185-191.
Donoghue, A. M. and Wishart, G. J. (2000) *Animal Reproduction Science,* **62,** 213-232.
Froman, D. P. (1997) In *Techniques for semen evaluation, semen storage, and fertility determination.*(Ed, Murray R. Bakst, H. C. C.) Poultry Science Association, Savoy, United States, pp. 56-59.
Fulton, J. (2006) *Poult Sci,* **85,** 227-231.
JUMP (2002) SAS Institute Inc.
Lake, P. E. (1968) In *VIth International Congress on Animal Reproduction and Artificial Insemination.*Paris, France. INRA, Jouy en Josas, pp. 1633-1635.
Long, J. (2006) *Poult Sci,* **85,** 232-236.
Marzoni, M., Castillo, A., Chiarini, R. and Romboli, I. (2003) *Italian Journal of Animal Science,* **2,** 184-186.
Tselutin, K., Seigneurin, F. and Blesbois, E. (1999) *Poult Sci,* **78,** 586-590.
Woelders, H., Zuidberg, C. and Hiemstra, S. (2006) *Poult Sci,* **85,** 216-222.