Research Notes

Cryopreservation of Rooster Semen in Thirteen and Sixteen Percent Glycerol

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ABSTRACT Semen from Barred Plymouth Rock roosters was cryopreserved with glycerol concentrations of 13 and 16% in a microprocessor-controlled freezer. Thawing and deglycerolation were facilitated by the use of an improved microprocessor-controlled thawing device and high speed dialyzer. Deglycerolated semen (100 μL; 192 and 154 million sperm, respectively, for the 13 and 16% glycerol concentration) was inseminated into Single Comb White Leghorn hens. Three inseminations were done at 4-d intervals. Eggs were collected for 10 d starting 1 d after the first insemination, and incubated for 4 d. Fertility was determined by candling after the 4th d. Fertility measurements of 62.4 and 65% were obtained from the sperm frozen in 13 and 16% glycerol concentrations, respectively, for the 10-d period.

(*Key words*: rooster, semen, cryopreservation, deglycerolation, insemination)

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INTRODUCTION

The potential impact of frozen semen on genetic selection programs in the poultry industry through the increased ability to select and use proven sires and to provide control for comparison of generations is recognized (Sexton, 1983; Lake, 1986; Hammerstedt, 1995; Reddy, 1995). Adoption of frozen semen technology by the poultry industry will require fertility that is satisfactory to the breeders using procedures that can be implemented at modest cost by unskilled personnel.

Success is inconsistent in most reports in the literature (Graham *et al.*, 1984; Lake, 1986; Hammerstedt and Graham, 1992). Persistent problems include the contraceptive effect of glycerol and damage to the thawed sperm during deglycerolation of semen. Although Van Voorst and Leenstra (1995a,b) reported 83 to 93% fertility after inseminating 692 to 425 million sperm in a single insemination, the sperm concentration used was two to three times higher than what is normally used in the industry. Buss (1993) reported a new method of freezing, thawing, and deglycerolating rooster semen that addressed the glycerol problem. He reported an average 9-d fertility of 55.6% with 120 to 180 million sperm per insemination. Experiments were conducted to test the protocol developed by Buss (1993) using improved instrumentation under conditions mimicking commercial operations.

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MATERIALS AND METHODS

Semen Collection and Freezing

Semen was collected two to three times a week by the method of Burrows and Quinn (1937) from a flock of 25 Barred Plymouth Rock (BPR) roosters from the same primary line used by Buss (1993). Birds were housed in individual cages (52.4 × 57.6 cm) under a day length of 18 h light (L):6 h dark (D), and fed a breeder ration. The samples were visually examined and those contaminated with fecal matter were discarded. Clean samples were cooled to 6 C by placing them in a bottle cooler. All the other materials required for processing the semen were placed in the bottle cooler to keep them at 6 C. Semen from the roosters was pooled, mixed, and divided into two equal portions. Each portion was diluted 1:2 with precooled Minnesota A (Tajima *et al.*, 1989) extender containing sufficient glycerol to give a final glycerol concentration of 13 or 16% (vol/vol). A small aliquot of the diluted semen was used for laboratory evaluation of semen characteristics. The remaining diluted semen was filled into cold (C) CellStor®[2] containers with a 1-mL syringe fitted with a 22-gauge needle, both the syringe and the needle were at 6 C. The fill holes of the containers were sealed with Dynamax light weld[3] ultraviolet light curing glue. The containers were quickly transferred to a microprocessor-controlled liquid nitrogen freezer[4] and frozen as described by Buss (1993). The frozen semen was immersed in liquid nitrogen until thawed for artificial insemination.

TABLE 1. Characteristics of rooster sperm before and after freeze-thaw in varying amounts of glycerol

Characteristic	Fresh semen	Thawed semen 13%	Thawed semen 16%
Count, 100 millions/100 μL	2.2 ± 0.1	1.9 ± 0.5	1.5 ± 0.4
Swirl, 0 to 4 scale	3.7 ± 0.2	4.0 ± 0	4.0 ± 0
Motility, %	66.9 ± 5.2	42.3 ± 6.7	60.0 ± 5.0
Hypo-osmotic swell test, % damaged	13.3 ± 2.3	46.0 ± 3.7	29.0 ± 2.6
Residual glycerol, %		0.3 ± 0.02	0.3 ± 0.02

Thawing Deglycerolation and Insemination

Fifteen containers representing each of the two glycerol concentrations were transferred from liquid nitrogen to vapor phase in a previously charged dry shipper[5] overnight. After charging with liquid nitrogen, the dry shipper maintained a temperature of –196 C. The dry shipper was carried to a small laboratory at the site of the chicken flock. The containers were transferred from the dry shipper to the microprocessor-controlled thawing device,[6] which automated the thawing process described by Buss (1993) and warmed the containers from –196 to 6 C. To remove the glycerol the containers were dialyzed in a high speed dialyzer.[7] The deglycerolation chamber of the dialyzer has a capacity for 20 containers. One thousand two hundred and fifty milliliters of the deglycerolation buffer (Beltsville Poultry Semen Extender) at 6 C was circulated past the containers at the rate of 7.6 L/min. After 120 min, the containers were removed from the deglycerolation chamber and placed in the bottle cooler at 6 C. Semen was removed from each container by puncturing two diagonal corners of the membrane and aspirating the contents with a 5-mL syringe and 22-gauge needle maintained at 6 C. The semen was pooled into cold 5-mL culture tubes and taken to the layer pens in a container of cold water (6 C). A small aliquot of the deglycerolated semen was removed for laboratory evaluation. The rest of the semen was inseminated (100 μL) into Single Comb White Leghorn (SCWL) hens with a Hamilton[8] syringe. Each hen was inseminated three times at 4-d intervals.

The SCWL hens were maintained under 9D:15L cycle with lights coming on at 0300 h. They were kept in serially numbered individual cages (30 × 45 cm) with nipple waterers and were fed standard layer feed. Only the hens having egg production rates of 80% or above were included in the experiment. One hundred hens each were inseminated with semen stored with 13 and 16% glycerol concentration. The inseminations were started at 1300 h and completed within 30 min of the deglycerolation of the respective groups. Eggs were collected beginning the 2nd d (Day 1) after the first insemination and continued for 10 d. Candling was done after 4 d of incubation with a 2AA cell Maglite.[9] Infertile and suspected fertile eggs were broken out in a pan of water to visually determine the signs of embryonic development.

Laboratory Analysis of Semen Quality

The aliquots of semen collected before the freeze and after the deglycerolation were evaluated immediately for motility (Buss, 1993), swirl (Cherms, 1968; Graham *et al.*, 1982) with microscope stage set at 31 C, sperm concentration (Taneja and Gowe, 1961; Wishart and Palmer, 1986), and the resistance during hypo-osmotic swell test (Bilgili and Renden, 1984; Bakst *et al.*, 1991). Residual glycerol concentration was determined (Buss, 1993) after deglycerolation. The data was subjected to factorial analysis as per Steel and Torrie (1980).

RESULTS AND DISCUSSION

Semen Quality

The average numbers of sperm (over three inseminations) inseminated into the hens per insemination were 192 ± 0.51, and 154 ± 0.39 million for the semen frozen at 13 and 16% glycerol concentration, respectively. They were higher than 1×10^9 per insemination. Wishart and Palmer (1986) have reported that using doses of 1×10^9 or greater provides a virtual saturation dose of sperm. There was no significant difference in the number of sperm inseminated into the hens among the three inseminations. The sperm concentration in the thawed semen was lower than the fresh semen. The swirl and motility of the deglycerolated semen samples were not significantly different among the inseminations (Table 1). Although the results were not significant, the hypo-osmotic swell test indicated increased sperm survival with higher glycerol concentration (Table 1).

Fertility

There was no significant difference in the average fertility over the period studied. However, fertility was

[5]MVE Model XC14/3V, Minnesota Valley Engineering Inc., New Prague, MN 56071.
[6]BioThaw, BioPore Inc., State College, PA 16805.
[7]BioStor, BioPore Inc., State College, PA 16805.
[8]Hamilton Co., Reno, NV 89520-0012.
[9]Maglite, Ridgefield, NJ 07657.

TABLE 2. Average fertility with frozen semen

Days	Glycerol concentration	
	13%	16%
	(%)	
1 to 4	58.4 ± 5.2	57.1 ± 4.6
5 to 8	61.8 ± 7.1	67.2 ± 4.8
9 to 10	71.4 ± 7.1	77.0 ± 2.3
1 to 10	62.4 ± 3.7	65.1 ± 3.4

lower in the first 4 d (58.4 and 57.1% for hens inseminated with semen cryopreserved in 13 and 16% glycerol containing diluent). It increased to 71.4 and 77% for the two groups on Days 9 and 10, respectively. The average fertility for the 10-d period was 62.4 and 65.1% for 13 and 16% groups, respectively (Table 2). Fertility obtained with frozen-thawed semen in this report confirms the results reported by Buss (1993). In this experiment, fertility was more than 10% greater than that previously reported by Buss (1993). Glycerol concentrations higher than those used by Buss (1993) were used to increase the cryoprotectant effect of glycerol. Semen cryopreserved at 16% glycerol concentration gave higher, but not significantly higher, fertility than that stored at 13% glycerol concentration. To minimize the suppression of fertility due to glycerol (Tajima *et al.*, 1989; Buss and Reddy, 1992; Buss, 1993), the dialysis time was increased from 90 to 120 min. This increase reduced the residual glycerol levels to between 0.2 and 0.3%. These results confirm the earlier findings of Buss (1993) and the current results of greater than 60% fertility over 10-d period may make this technique viable for adoption by the industry. However, line differences (Marini and Goodman, 1969) in semen quality would be expected in the commercial lines and this could result in lower or higher fertility being obtained in the industry with cryopreserved semen. Better fertility obtained in this experiment could be attributed to the use of higher glycerol concentration and the use of improved instrumentation that reduced temperature fluctuations during various stages of semen processing.

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