Cryopreservation of Semen from Onagadori, the Japanese Long-Tailed Fowl

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Semen freezing is considered a valuable method for long-term preservation of fowl spermatozoa (see review by Sexton, 1979). It, therefore, seemed reasonable to ascertain whether such procedures would be suitable with the Japanese Long-Tailed (L-T) Fowl semen. In this case long-term preservation of semen would be important because the L-T Fowl has significant physiological and historical traits. The L-T cocks are unique in that they never molt 20 of their 40 tail, sickle, covert, mutation, and lining feathers (Oana, 1954). This phenomenon remains unexplained by avian scientists. Efforts to aid in the reproduction of the L-T Fowl are of great interest to the Japanese Government since this breed is a Natural Commemorated Object (Ogasawara, 1979). The existence of frozen semen banks would also aid the L-T Fowl breeders of both Japan and the United States.

The specific objectives of this study were to determine the optimum pre-freeze technique (dilution, cooling time, level of dimethylsulfoxide (DMSO) and equilibration time) and freezing method which would result in the highest post-thaw motility recovery of spermatozoa from the L-T Fowl.

Materials and Methods

Ten L-T Fowl males served as semen donors and were housed in a facility maintained at 23°C and with a 14L:10D lighting schedule. The birds were fed *ad libitum* a 15.8% protein breeding ration except that feed was withheld 15 to 18 h prior to the collection of the semen. The semen volume was recorded for each pooled sample.

Semen was collected on Monday, Wednesday, and Friday of each week using the method of Burrows and Quinn (1935). Two ejaculates were collected from each male and pooled.

Six independent trials were conducted sequentially to determine optimal processing techniques for L-T Fowl semen. Aspects of the procedure studied were dilution rate, holding time at 5°C in both the presence and absence of DMSO and various freeze rates. The pre-freeze (Steps 1 to 5) and freezing (Step 6) procedures tested are presented in Table 1, column 1. Once an optimal parameter (Table 1, columns 2 and 3) was determined, it was used in the sequential trials for evaluation of subsequent treatments.

For the freezing process, polypropylene straws were filled with the pre-freeze treated semen. The filled straws were placed in plastic goblets attached to freezing canes. The canes were placed in a plastic-constructed holder and this apparatus was set in 1500 ml of

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Table 1. The effect of various pre-freeze and freeze procedures on the motility of Long-Tailed Fowl spermatazoa.

Procedure	Parameter determined	Optimal parameter	Motility ^a score
1. Semen collection	Volume, concentration & motility	—	4.5±0.1
2. Dilute semen with BPSE	Semen : Extender (1 : 1, 1 : 3, 1 : 5)	1 : 3	4.5±0.3
3. Hold semen at 5°C	Time-Hr (0.5, 1.0, 1.5, 2.0)	1.0 hr	4.2±0.2
4. Add DMSO in bulk	Concentration-% (2, 4, 6)	4%	4.2±0.2
5. Equilibrate semen and DMSO at 5°C	Time-Hr (0.5, 1.0, 1.5, 2.0)	1.0 hr	4.3±0.2
6. Freeze from			
a. +5°→-20°C before storage in liquid nitrogen	Rate-°C/min (0.5, 1.0, 5.0)	1.0°C/min	1.0±0.0
b. -20°→-81° to -154°C before storage in liquid nitrogen	Liquid nitrogen vapor temperature (-81° to -154°)	-110°C	3.5±0.0
7. Thaw at 2 to 3°C	—	—	—

^a Mean and standard error of the mean.

refrigerated (5°C) alcohol. A Linde Biological Control Rate Freezer was used to produce various freeze rates from +5°C to -20°C (Table 1, Step 6a). A continuous and permanent record of changes in semen temperature was accomplished with a thermo couple and Honeywell Elektronik Recorder. When the semen temperature reached -20°C, the samples were placed in a specially constructed Dewar flask. Attached to the lid of the Dewar flask was a rod and wire rack where the semen canes in goblets and a thermocouple were placed. Semen samples were exposed to various intermediate liquid nitrogen vapor temperatures (Table 1, Step 6b) by altering the level of liquid nitrogen in the flask and/or by varying the level of the rack above the liquid nitrogen. The samples were held in the vapor for 10 min prior to plunging into liquid nitrogen (-196°C) and thawed in a 2 to 3°C ice bath.

The semen volume was recorded for each pooled sample. The motility of the spermatazoa was observed to evaluate the effects of steps in the pre- and post-freeze procedures on cell quality survival. Motility scoring was a modified version of that reported by Allen and Champion (1955); a zero to five scale was used, with five indicating the most motile spermatazoa. Spermatozoal concentration was determined with a Klett-Summerson photoelectric colorimeter, standardized with hemacytometer counts, according to the modified method of Carson *et al.* (1955).

Results and Discussion

The average volume of semen collected for the trials from the 10 males was 1.34 ml with an average of 0.12 ml per ejaculate. The mean concentration of spermatazoa was 4.25 billion spermatazoa per ml of semen with an average motility score in freshly collected semen of 4.5.

A composite summary of the optimal pre-freeze methods is outlined in Table 1. The highest motility scores were observed when semen was diluted 1:1 or 1:3 (semen:extender)

when compared to samples diluted 1:5, i. e. 4.8 ± 0.3 and 3.0 ± 0.0 , respectively. Little difference was noted in motility scores (4.0 to 4.2) of semen diluted 1:3 and equilibrated at 5°C for 0.5, 1.0, 1.5 or 2 h. The addition of 2 or 4% DMSO caused the least decline in pre-freeze motility (4.0 ± 0.3 and 4.2 ± 0.2) while the addition of 6% DMSO resulted in a larger drop in motility (3.8 ± 0.3). The time for which diluted semen was equilibrated with DMSO had little consequence on the motility of semen diluted 1:3 and cooled 1 h at 5°C before addition of 4% DMSO. The motility scores for samples equilibrated at 5°C for 0.5, 1.0, 1.5 or 2 h were 4.2, 4.3, 4.2, and 4.0, respectively. A similar approach was used to assess the effect of pre-freeze treatments on the post-thaw motility of crane and chicken spermatozoa (Sexton and Gee, 1978a). It is apparent that there are species differences in the motility response of spermatozoa to certain prefreeze treatments, but because of the limited scope of this study it is difficult to explain the differences.

The response of L-T Fowl spermatozoa to various freeze rates from +5° to -20° is similar to that reported for chicken spermatozoa (Lake, 1978; Sexton, 1978b). Post-thaw motility scores (1.0 ± 0.0) were highest when semen was cooled from +5 to -20° at a rate of either 0.5 or 1.0°C/min. The highest recovery of motility (3.5) was obtained when the semen was further cooled from -20°C to -110°C in liquid nitrogen vapor before plunging into liquid nitrogen. Similar freeze rates were determined recently to be optimal for chicken semen (Sexton, 1980).

The first hatch of L-T Fowl chicks from eggs fertilized with semen frozen according to the procedure outlined in Table 1 was recently reported (Bradley and Ogasawara, 1980). The overall fertility was low (12%), but it is hoped that further modification of the technique using the information gathered in the present study will improve fertility.

Summary

Six independent trials were conducted sequentially with semen from the Japanese Long-Tailed Fowl to determine the optimal pre-freeze and freeze procedures for maximum recovery of spermatozoal motility. Highest levels of pre-freeze motility (4.3 ± 0.2) were obtained when semen was diluted 1:3 with Beltsville Poultry Semen Extender, cooled for 1.0 h at 5°C, then 4% dimethylsulfoxide (DMSO) added in bulk, and the sample equilibrated an additional 1.0 h at 5°C. Highest levels of post-thaw motility (3.5 ± 0.0) were obtained when semen was first frozen at 1.0°C/min from +5°C to -20°C, then exposed to liquid nitrogen vapor temperatures between -81° and -154°C for 10 min, before plunging into liquid nitrogen.

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尾 長 鶏 精 液 の 凍 結 保 存

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尾長鶏の精液について、最高の生存率を得るための凍結前処理法および凍結処理法に関する 6 回の連続実験を行った。その結果、精液をベルツビル家禽精液希釈液で希釈 (1:3) して 1 時間 1°C 下におき、これに 4% 容量となるよう ジメチル 酸化硫黄を加えて、さらに 1 時間 5°C 下におき平衡状態としたとき、最高レベルの凍結前

生存率 (生存指数 4.3 ± 0.2) が得られた。また、精液を液体窒素中に投入する前に、先ず +5°C から -20°C まですで毎分 1°C の割合で冷却してから液体窒素ガス (-80°C ~ -154°C) に 10 分間さらすと、融解後の生存率は最高レベルの値 (生存指数 3.5 ± 0.0) を示した。

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