J. Fac. Fish. Anim. Husb.
Hiroshima Univ. (1972), **11**:33~37

Freeze-drying of Fowl Spermatozoa

I. Diluents for Freeze-drying of Fowl Spermatozoa

Moriyuki WATANABE and Shuichi KATO

Department of Animal Husbandry, Faculty of Fisheries and Animal Husbandry
Hiroshima University, Fukuyama

(Figs. 1-2, Table 1; Plate 1)

Since POLGE *et al.* (1949)[1] investigated for the first time the effects of freeze-drying on fowl semen as a method for long-term semen preservation, increased attention has been given to techniques for freeze-drying bovine spermatozoa. In 1959, MERYMAN and KAFIG[2] reported that they had carried out the freeze-drying of bovine spermatozoa through the courtesy of the Department of Dairy Science of the University of Maryland and later on gained the normal pregnancy and delivery of a normal heifer calf. However, in their further experiments they (1963)[3] were no longer able to recover motile spermatozoa under any circumstances. They concluded that the experiment should be postponed pending to the development of a better understanding of the mechanisms of freezing and drying injury in general.

The present experiments were conducted in order to determine the several diluents, with or without the addition of glycerol, influencing the recovery.

MATERIALS AND METHODS

The cocks used for the collection of semen were of one to three year old Single Comb White Leghorns. For collecting the semen the "Hiroshima" pattern bird holder, deviced by YAMANE *et al.* (1962),[4] was used. The semen samples were collected at. 8.00 to 8.30 a. m. every three or four days by the abdominal massage method. The order of the freeze-drying methods employed in the present experiment are shown in Fig. 1. Namely the collected fowl semen were diluted at 37°C to three fold with such solutions as whole milk, egg yolk citrate solution (EYC), gelatin, egg yolk glucose (EYG) and glycerol egg yolk glucose (GEYG) solutions. The components of every diluent used for this experiment are shown in Table 1. The diluted semen was taken up on a sheet of nylon gauze roughly 2×2 cm in dimension with a 0.25 mm mesh. This gauze was immediately hung on the supporting rack in a separable flask of 13.5 cm height and 7.5 cm in diameter which was connected through a stopcock with a 12 mm bore to the inlet of a mechanical vacuum pump with a free-air pumping speed of 50 *l*/min. as shown in Fig. 1 and Plate 1. The ultimate vacuum of the mechanical vacuum pump used was 5×10^{-4} mmHg. The trap was maintained at -196°C by one litre nitrogen liquid. After

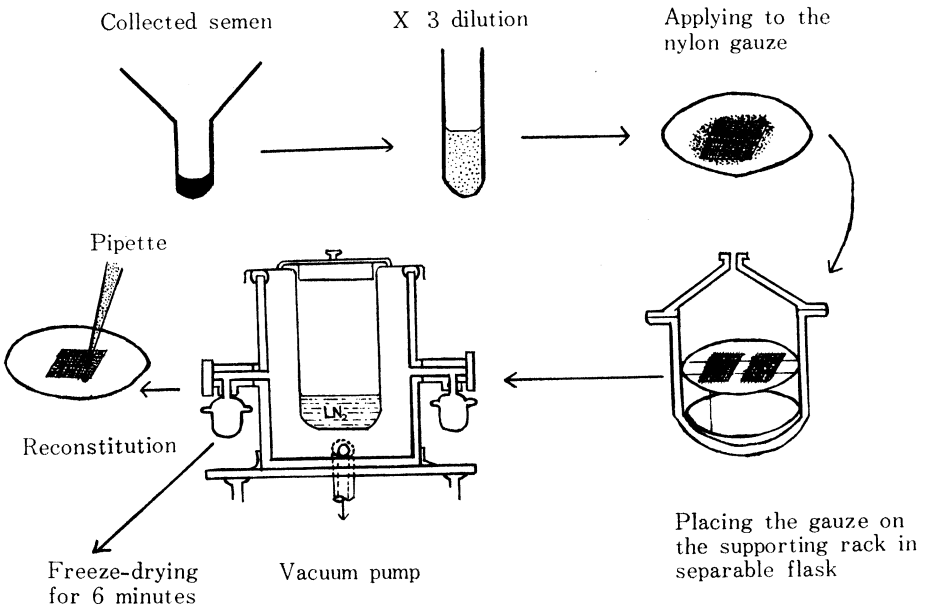


Fig. 1. Order of the freeze-drying method.

Table 1. The components of diluents used for this experiment.

Diluent	Components
Whole milk	Hot sterilization for 10 minutes at 90~95°C
EYC solution	22% Egg yolk, 2.9% Sodium citrate
Gelatin	5.0% Gelatin, 4.2% Glucose
EYG solution	15% Egg yolk, 4.2% Glucose
GEYG solution	9.1% Glycerol, 13.7% Egg yolk, 3.8% Glucose

the gauze was hung in the separable flask, the vacuum valve opened slowly. The diluted semen was routinely freeze-dried for 6 minutes and reconstituted immediately with the same extender of 0.05 m*l*. The diluted, reconstituted semen was held at 37°C and the motility of those semen were examined.

RESULTS AND DISCUSSION

The results of the present experiment are shown in Fig. 2. The spermatozoa in semen samples diluted with whole milk, egg yolk citrate solution and gelatin, which were freeze-dried for 6 minutes and then reconstituted immediately with the same extender of 0.05 m*l* respectively, did not survive at all. The spermatozoa in semen sample with egg yolk glucose solution followed by freeze-drying for 6 minutes

and reconstituting with the same extender also failed with the exception of one single experiment which the motility showed 10 percent (over ++) after reconstitution. In the semen samples diluted with glycerol egg yolk glucose solution, motile spermatozoa were observed at every experiment and the spermatozoa in some experiment showed 20 percent (over ++) motility.

The percentage of motile spermatozoa recovered averaged 10 percent in the

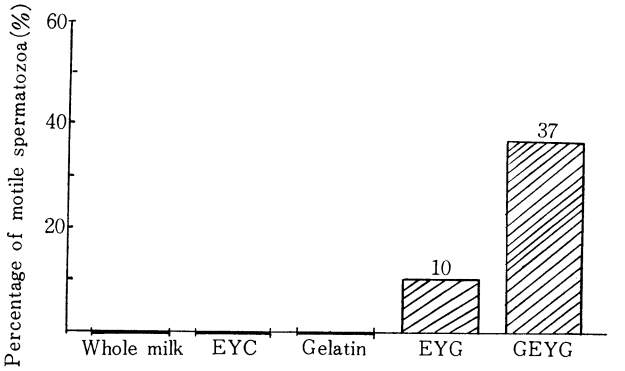


Fig. 2. Percentage of motile spermatozoa appearing in the semen following reconstitution.

semen samples diluted with egg yolk glucose and averaged 37 percent in the semen samples diluted with glycerol egg yolk glucose solution as shown in Fig. 2. From the result of the present experiment, it would appear that the most adequate preservation medium for withstanding freeze-drying was glycerol egg yolk glucose solution. The amount of water removed during the freeze-drying process in this study was not measured. According to POLGE *et al.* (1949),[1] about 90 percent of the original water was removed when a distillation process of high vacuum against liquid air was used to desiccate fowl semen, diluted with 30 percent glycerol in Ringer's solution. BIALY and SMITH (1957)[5] observed negative results when the dehydration level exceeded 71 percent in freeze-drying bovine semen. LEIDL (1956)[6] also reported that he observed living bull spermatozoa following freeze-drying and reconstitution when as long as the final water content did not go lower than 5 percent and the glycerol content did not exceed 50 percent. Judging from the reports of the above mentioned authors, the rate of water removal as measured by weight change during freeze-drying seems to be a exceedingly important factor for recovery of progressively motile spermatozoa following freeze-drying and reconstitution. Thus, the diluents or extenders, the length of freeze-drying time of spermatozoa and the total water content remaining in sperm cell have to be studied furthermore. Also the manipulation of semen smear will need further examination.

SUMMARY

The present experiment was undertaken in order to determine the diluents suitable for the freeze-drying and reconstitution of fowl spermatozoa. Whole milk, egg yolk citrate solution, gelatin, egg yolk glucose and glycerol egg yolk glucose solution were used for the extender of semen. The results are as follows;

1. In the semen samples diluted with glycerol egg yolk glucose solution, motile spermatozoa were observed at every experiment and the spermatozoa in some case showed 20 percent motility (over ++) and the percentage of motile spermatozoa recovered averaged 37 percent.
2. In the semen samples diluted with egg yolk glucose solution following freeze-drying and reconstitution, motile spermatozoa were not observed with the exception of one single experiment which the motility showed 10 percent (over ++) and the percentage of motile spermatozoa recovered averaged 10 percent.
3. In semen sample diluted with the other three diluents, i. e., whole milk, egg yolk citrate solution and gelatin, motile spermatozoa were not observed at all.
4. Therefore, out of above 5 diluents glycerol egg yolk glucose solution seemed to be the most adequate medium for withstanding freeze-drying preservasion of fowl spermatozoa.

REFERENCES

1) POLGE, C., SMITH, A. U., and PARKES, A. S.,: *Nature,* **164**, 666 (1949).
2) MERYMAN, H. T., and KAFIG, E.: *Nature,* **184**, 470–471 (1959).
3) MERYMAN, H. T., and KAFIG, E.: *J. Reprod. Fertil.,* **5**, 87–94 (1963).
4) YAMANE, J., TSUKUNAGA, S., and TAKAHASHI, T.: *Bull. Hiroshima Agric. Coll.,* **2**, 17–24 (1962).
5) BIALY, G., and SMITH, U. R.: *J. Daily Sci.,* **40** 739–745 (1957).
6) LEIDL, W.: Proc. 3rd Intern. Congr. Anim. Prod., Cambridge, **3**, 39 (1956).

鶏精子の凍結乾燥に関する研究

Ⅰ．鶏精子の凍結乾燥用保護媒質について

渡辺守之・加藤秀一

鶏精子の凍結乾燥用保護媒質として全乳，卵ク液，ゼラチン，卵ブ液およびグリセリン卵ブ液を用いて実験を行なった結果は次の如くである．

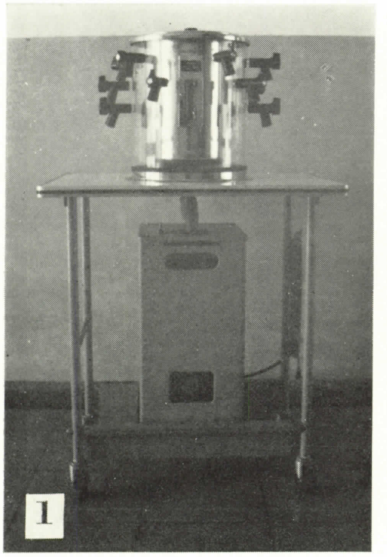
1．グリセリン卵ブ液を保護媒質とした精液では復元後何れの場合も活力のある精子が観察され，或る実験では活力++以上を示すものが20%もあり，運動力のある精子の出現割合は平均37%であった．

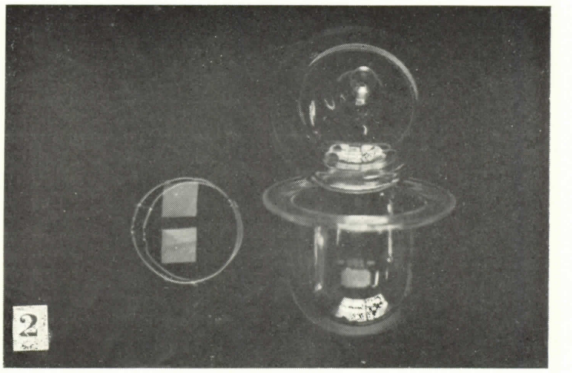
2．卵ブ液を保護媒質とした精液では活力++以上を示すもの10%の1例を除いては運動精子は観察されず，その出現割合は平均10%であった．

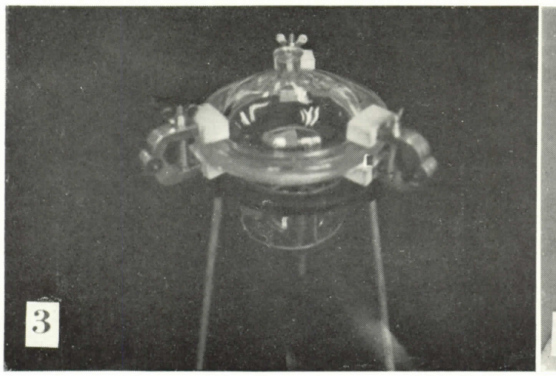
3．全乳，卵ク液およびゼラチンを保護媒質とした精液では復元後全く運動精子が観察されなかった．

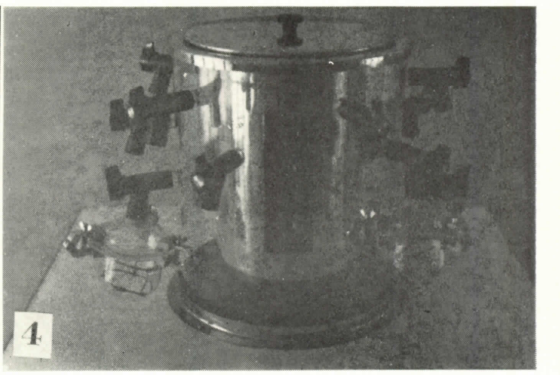
4．現在の処5つの保護媒質の中でグリセリン卵ブ液が最も適した保存液のように思われる．

Plate 1









Explanation of plate

Fig. 1. DC31-Model equipment for freeze-drying made by Yamato Kagakukikai.
Fig. 2. Nylon gauze on the supporting rack and the separable flask.
Fig. 3. Nylon gauze kept in separable flask.
Fig. 4. Separable flask connected through a stopcock with a 12 mm bore to the inlet of a mechanical vacuum pump.