J. Fac. App. Biol. Sci.,
Hiroshima Univ. (1985), 24: 25–31

Studies on Morphological Frozen-Injuries of Avian Spermatozoa: Optimum Glycerol Concentration for Freezing Fowl Spermatozoa

Teruo MAEDA, Hiromi SENBA, Takato TERADA and Yoshio TSUTSUMI

Animal Reproduction Laboratory, Faculty of Applied Biological Science, Hiroshima University, Fukuyama

Received: August 21, 1985

INTRODUCTION

In our previous experiment[1], the effects of various cryoprotectants (glycerol, dimethylsulphoxide (DMSO), methylformamide and ethylene glycol) in the same molarity on morphological preservation of frozen-thawed spermatozoa were examined under a light and scanning electron microscope (SEM) to find out the most suitable cryoprotectant. It was concluded that glycerol was superior to ethylene glycol, DMSO and methylformamide as a cryoprotectant for fowl spermatozoa. It has also been proved in the experiments on bull[2, 3], ram [4, 5] and boar[6] spermatozoa that the cryoprotective effect of glycerol was most useful.

The optimum glycerol concentration for freezing spermatozoa of bulls [7–9], rams[10,11], boars[6] and fowls[12] has been determined mainly by motility or survival of spermatozoa after frozen-thawed. POLGE [13] and GRAHAM *et al.*[14], however, suggested that there is poor correlation between the spermatozoal motility and fertility in boars in the presence of glycerol, and that the morphological observation of frozen-thawed spermatozoa is important, especially in acrosome and midpiece. It is also reported that there is high correlation in fowl between the crooked-necked spermatozoa and the fertilizing ability[15]. These results suggest that the inspection of spermatozoal structure is as a reliable method for assessment of fertilizing ability of frozen-thawed spermatozoa as the evaluation of motility or survival of spermatozoa. Thus, the acrosomal integrity or the acrosomal deterioration was investigated to determine the optimum glycerol concentration for freezing semens of mammals (bulls[16,17], rams[18] and boars[6]). As for avian frozen spermatozoa, however, there are no reports on determination of the optimum glycerol concentration based on the morphological integrity of spermatozoa, probably because of their small size[19–21].

In the present study, morphological and physiological observations were conducted to determine the optimum glycerol concentration for freezing fowl spermatozoa, especially focussed on the injuries of the acrosome and midpiece through the freeze-thawing procedures.

MATERIALS AND METHODS

Collection and treatment of semen

Semen was collected from 10 White Leghorn cocks (10 to 15 months old), housed in an individual cage, by the abdominal massage technique[22], and pooled in the glass centrifuge tube which was placed in a beaker containing water at 5°C. One volume of semen was mixed with three volumes of 5.7% glucose solution which contained 0, 5, 7, 10, 15 and 20% glycerol, respectively. Each diluted semen (0.2 mℓ) was frozen in a pelleted form[23] after 2 – 10 min. glycerol equilibration. The cooling rate of semen samples from +5 to −60°C was 27°C/min. Five frozen pellets in a 10 mℓ-test tube were thawed in a water bath at 37°C after 1–2 hour storage at −79°C. Each frozen-thawed semen sample was subjected to the following three procedures.

Estimation of sperm motility

Sperm motility of frozen-thawed semen was estimated on a microscope stage incubator at 37°C, and scored according to the method described by CLARK and SHAFFNER[24]. Units used for scoring ranged from five (maximum motility) to zero (completely inactive spermatozoa).

Treatment for light-microscopic examination

Postthawed semen was smeared on a glass slide. The smears were fixed in formaline vapor for 1 hour at 5°C, and air-dried, and then dipped into an alcohol (> 95%) bath for 10 min. to remove alcohol soluble contaminants, and air-dried again. The incidence of crooked-necked spermatozoa (CNS) was calculated approximately at 500 cells under a phase-contrast microscope (PCM).

Treatment for scanning-electron-microscopic examination

Spermatozoa in postthawed semen were fixed at 5°C for 1 hour by mixing with 25% glutaraldehyde solution to the final concentration of 3%. The solution suspended while the fixed spermatozoa were centrifuged at 700 g for 10 min. at 5°C, and then the supernatant was discarded. The precipitated spermatozoa were washed twice with a phosphate buffer solution[25] by centrifugation (700 g, 10 min.), and finally resuspended in the buffer solution. A drop of the sperm suspension was mounted on an aluminum foil. The foil was slanted, so that a smear of spermatozoa could be made by removing superfluous suspension with a paper filter. The smears on the foil were air-dried, and dehydrated in a series of ethanol-water mixtures, and air-dried again, and then gold-coated and examined with SEM (JEOL, JSM-T20). As for the incidence of abnormal acrosome (acrosomal deterioration), about 500 spermatozoa were inspected by SEM.

Statistical analysis

Data (incidences of CNS and acrosomal deterioration) were transformed to angles for

statistical treatment, and the significant differences between means were determined by use of DUNCAN's multiple range test [26].

RESULTS

Each of the motility of frozen-thawed semen treated with the six different concentrations of glycerol is shown in Table 1. The motility of samples without glycerol was zero. The highest motility was obtained at 10% glycerol, and the significant difference ($p < 0.05$) exists between 10% and the other four glycerol groups, 0, 5, 15 and 20%. Moreover, the motility of 7% glycerol group is significantly higher ($p < 0.05$) than that of 0 and 20% group.

Most of abnormal spermatozoa found by PCM in all the samples were CNS damaged at the midpiece. As shown in Table 1, there was a tendency to increase in the incidence of CNS with the increase of glycerol concentration, though no significant differences were recognized between the samples 0 to 15% glycerol concentrations. The incidence of CNS at 20% glycerol was significantly higher in comparison with that of CNS at 0, 5 and 7% glycerol, respectively ($p < 0.05$).

Table 1. Effects of glycerol on the motility and incidence of crooked-necked spermatozoa (CNS) in frozen-thawed fowl semen

Glycerol concentration (%)	Motility[1)]	Incidence of CNS[1)] (%)
0	0 ± 0^{a}	3.5 ± 2.2^{a}
5	3.1 ± 0.4^{b}	5.1 ± 1.8^{a}
7	3.8 ± 0.3^{bc}	4.5 ± 1.5^{a}
10	4.1 ± 0.7^{c}	7.5 ± 4.0^{ab}
15	3.0 ± 1.0^{b}	7.8 ± 2.2^{ab}
20	2.1 ± 0.6^{d}	11.6 ± 6.1^{b}

1) Mean ± standard deviation from 5 trials.
a,b,c,d Means with different superscripts within the same column are significantly different ($p < 0.05$).

The acrosome, nucleus, midpiece and tail of spermatozoa were clearly identified by SEM. CNS's, which were crooked at the neck region or through the full length of the midpiece, were checked in this observation on careful comparison with those in PCM observation. No morphological differences due to the different concentrations of glycerol, however, were noticed in CNS after frozen-thawed, and the similar tendency was recognized in the incidence of CNS.

The normal acrosome is conical and has a smooth surface. Various kinds of abnormal acrosomes, such as separated, swollen, detached, rough and disintegrated one, however, were found in all the frozen-thawed semen samples (see our previous reports[1,21]). There were no differences in the morphology of abnormal acrosomes of frozen-thawed spermatozoa treated with the different concentrations of glycerol.

Table 2. Effects of glycerol on the incidence of acrosomal deterioration in frozen-thawed spermatozoa

Glycerol concentration (%)	Acrosomal deterioration[1)]			
	Separation (%)	Swelling (%)	Other abnormality (%)	Total (%)
0	1.2 ± 1.1[a]	3.0 ± 1.9[a]	5.8 ± 1.9[a]	10.0 ± 4.1[ab]
5	0.8 ± 0.7[a]	2.1 ± 0.5[a]	3.6 ± 1.2[bc]	6.5 ± 2.0[ab]
7	0.7 ± 0.7[a]	2.2 ± 1.1[a]	2.6 ± 0.7[b]	5.5 ± 2.1[a]
10	0.8 ± 0.8[a]	2.4 ± 0.6[a]	4.7 ± 1.3[abc]	7.9 ± 2.0[ab]
15	1.2 ± 1.1[a]	4.1 ± 3.0[a]	5.2 ± 1.5[ac]	10.6 ± 4.8[b]
20	1.2 ± 1.0[a]	2.7 ± 0.4[a]	5.7 ± 1.3[ac]	9.6 ± 2.1[ab]

[1)]Mean ± standard deviation from 5 trials.

[a,b,c]Means with different superscripts within the same column are significantly different ($p < 0.05$).

Table 2 shows the effects of glycerol concentration on the incidence of abnormal acrosomes (acrosomal deterioration), which were classified into three types: 1, separation of acrosomes; 2, swelling of acrosomes; and 3, other abnormalities. In type 1, the incidence of abnormalities in the glycerol groups did not differ significantly each other. The incidence of type 2 ranged from 2.1 to 4.1%, and no significant differences were found. In type 3 the incidences decreased from 5.8 to 2.6%, with the increase of glycerol concentration from 0 to 7%, but they increased gradually from 4.7 to 5.7% according to the increase of glycerol concentration from 10 to 20%. As for the total acrosome deterioration, the 7% glycerol group showed the lowest value, and a significant difference ($p < 0.05$) exists between this and the 15% glycerol group.

Some abnormal acrosomes were observed in both CNS and normal formed spermatozoa. It could not be proved whether or not a great deal of spermatozoa with acrosomal damage always occur in CNS.

DISCUSSION

ROBBINS *et al.* [16] reported that bull spermatozoa were affected in the structure by glycerol concentration exceeding 10% in the diluted semen, and suggested that the optimum concentration of glycerol for preventing most effectively morphological injuries by freeze-thawing was 8.5%. Similarly, it was insisted on by BECKER *et al.* [17] that the optimum concentration of glycerol for acrosomal maintenance was 7–9% in frozen bull spermatozoa. WATSON and MARTIN [18] reported that glycerol concentration over 7.5% had a detrimental effect on the acrosomal structure, and a lower concentration than 7% might be advantageous in frozen-thawed ram spermatozoa. Boar spermatozoa were more sensitive to glycerol, and tended to be influenced detrimentally, and low glycerol concentration was favorable for freezing the spermatozoa[6]. More evidence of sperm membrane damage by freeze-thawing was demonstrated by the increased release of glutamic oxalacetic transaminase from boar spermatozoa when glycerol concentration was increased to 8%[27]. Thus, it is clear that the sensitivity of spermatozoa to glycerol

toxicity varies according to species, while glycerol is cryoprotective in spite of its cytotoxic activity especially to the acrosomal region.

In our present experiments, the incidence of CNS (Table 1) and acrosomal deterioration (Table 2) in frozen-thawed fowl semen became greater at the glycerol concentration over 10%. Therefore, it appears that higher concentration of glycerol makes severe morphological injuries in fowl spermatozoa as well as in bull, ram and boar spermatozoa. Several studies have been conducted to determine the effects of glycerol on the ultrastructure of chicken and turkey spermatozoa. It can be suggested that ultrastructural damages of spermatozoa may be cuased by the treatment of glycerol[28-30].

The incidence of CNS in frozen-thawed fowl semen diluted with the solution containing 0 to 7% glycerol was comparatively lower, though no motile spermatozoa were observed at 0% glycerol, and the motility at 5% glycerol was poor (Table 1). Acrosomal deterioration was also relatively low at 7% glycerol. Sperm motilities and oxygen consumption rates of frozen-thawed fowl semen diluted with the solution containing different concentrations of glycerol were maximum in 7 and 10% glycerol concentration, respectively[31]. MASUDA *et al.*[12] remarked that the optimum glycerol concentration was 7% (ranged from 6 to 9%) based on the examination of the effects of glycerol concentration on the survival of frozen-thawed fowl spermatozoa. Therefore, it may be concluded that the optimum glycerol concentration for freezing fowl spermatozoa is about 7%. The optimum glycerol concentration, however, may be influenced by the component of diluent, freezing rate, thawing rate, freezing methods, and so on [17, 18]. Further studies on the effects of various factors on the morphological frozen-injuries will be necessary. Our studies on the correlation between abnormal acrosome and CNS in frozen-thawed fowl spermatozoa are in progress.

SUMMARY

In the present study, morphological and physiological observations were conducted to examine the effects of glycerol concentration and to determine the optimum concentration for frozen-thawed fowl spermatozoa, especially focussed on the injuries of the acrosome and midpiece of spermatozoa.

The highest motility was obtained at 10% glycerol, and the secondary was at 7%. The incidence of crooked-necked spermatozoa (CNS) and acrosomal deterioration in frozen-thawed semen samples were greater in higher glycerol concentrations. Both incidence of CNS and acrosomal deterioration in frozen-thawed semen diluted with the solution containing 7% glycerol were relatively low in all the concentrations of glycerol.

These results may suggest that the morphological injuries increase with the increase of glycerol concentration, and that the optimum glycerol concentration for frozen-thawed fowl spermatozoa is about 7%.

REFERENCES

1) MAEDA, T., TERADA, T., and TSUTSUMI, Y. : *Br. Poult. Sci.,* **25**, 547–553 (1984).
2) NAGASE, H. : *In VI Int. Congr. Anim. Reprod. A. I. (Paris)* 2, 1103–1105 (1968).
3) NAGASE, H., and TOMIZUKA, T. : *In VI Int. Congr. Anim. Reprod. A. I. (Paris)* 2, 1107–1109 (1968).
4) JONES, R. C. : *Aust. J. Biol. Sci.,* **18**, 877–885 (1965).
5) SALAMON, S. : *Aust. J. Biol. Sci.,* **21**, 351–360 (1968).
6) WILMUT, I., and POLGE, C. : *Cryobiology*, **14**, 471–478 (1977).
7) RODRIGUEZ, O.L., BERNDTSON, W. E., ENNEN, B. D., and PICKETT, B. W. : *J. Anim. Sci.,* **41**, 129–136 (1975).
8) MORTIMER, R. G., BERNDTSON, W. E., ENNEN, B. D., and PICKETT, B. W. : *J. Dairy Sci.,* **59**, 2134–2137 (1976).
9) UNAL, M. B., BERNDTSON, W. E., and PICKETT, B. W. : *J. Dairy Sci.,* **61**, 83–89 (1978).
10) FIRST, N. L., HENNEMAN, H. A., MAGEE, W. T., and WILLIAMS, J. A. : *J. Anim. Sci.,* **20**, 74–78 (1961).
11) FISER, P. S., and FAIRFULL, R. W. : *Cryobiology,* **21**, 542–551 (1984).
12) MASUDA, H., SOEJIMA, A., and WAIDE, Y. : *Bull. Nat. Inst. Anim. Ind.,* **28**, 33–40 (1974).
13) POLGE, C. : *J. Reprod. Fert.*,**54**, 461–470 (1978).
14) GRAHAM, E. F., RAJAMANNAN, A. H. J., SCHMEHL, M. K. L., MAKI-LAURILA, M., and BOWER, R. E. : *A. I. Digest,* **19**, 6–16 (1971).
15) SAEKI, Y. : *Poult. Sci.,* **39**, 1354–1361 (1960).
16) ROBBINS, R. K., SAACKE, R. G., and CHANDLER, P. T. : *J. Anim. Sci.,* **42**, 145–154 (1976).
17) BECKER, W. C., SENGER, P. L., AALSETH, E. P., and MARSHALL, C. E. : *J. Anim. Sci.,* **44**, 1067–1071 (1977).
18) WATSON, P. F., and MARTIN, I. C. A. : *Aust. J. Biol. Sci.,* **28**, 153–159 (1975).
19) MARQUEZ, B. J., and OGASAWARA, F. X. : *Poult. Sci.,* **54**, 1139–1143 (1975).
20) THURSTON, R. J., HESS, R. A., HUGHES, B. L., and FROMAN, D. P. : *Poult. Sci.,* **61**, 1738–1743 (1982).
21) MAEDA, T., TERADA, T., and TSUTSUMI, Y. : *Br. Poult. Sci.,* **25**, 409–413 (1984).
22) BOGDONOFF, P.D., and SHAFFNER, C. S. : *Poult. Sci.,* **33**, 665–669 (1954).
23) TERADA, T. : *Jap. Poult. Sci.,* **20**, 15–20 (1983).
24) CLARK, C. E., and SHAFFNER, C. S. : *Poult. Sci.,* **39**, 1213–1220 (1960).
25) WILCOX, F. H., and SHAFFNER, C. S. : *Poult. Sci.,* **37**, 1353–1357 (1958).
26) DUNCAN, D. B. : *Biometrics,* **11**, 1–42 (1955).
27) BOWER, R. E., CRABO, B. G., PACE, M.M., and GRAHAM, E. F. : *J. Anim. Sci.,* **36**, 319–324 (1973).
28) WESTFALL, F. D., and HOWARTH, B., JR. : *Poult. Sci.,* **56**, 1454–1456 (1977).
29) MARQUEZ, B. J., and OGASAWARA, F. X. : *Poult. Sci.,* **56**, 1806–1813 (1977).
30) BAKST, M. R., and SEXTON, T. J. : *J. Reprod. Fert.,* **55**, 1–7 (1979).
31) TERADA, T., MAEDA, T., WATANABE, M., and TSUTSUMI, Y. : *Poult. Sci.,* **62**, 2271–2275 (1983).

家禽精子の凍結障害に関する研究：鶏凍結精子に対する最適グリセリン濃度について

前田照夫・仙波寛美・寺田隆登・堤　義雄

家禽精子の凍結障害に関する生理・形態学的一連の研究の一つとして，本実験では種々の濃度のグリセリンを含むブドウ糖溶液で凍結処理した鶏精子の運動性，首曲がり精子率および先体異常率を調べ，グリセリンの最適濃度を検討した。

融解後の精子の運動性は10％グリセリン濃度区で最も高く，次いで7％区であったが，両区間には有意差は認められなかった。また0％区では運動精子は観察されなかった。

融解後の首曲がり精子率は0および7％区で低い値を示し，7％以上の区では濃度の上昇に伴い高くなる傾向が認められた。先体異常率は7％区で最も低く，7％区と15％区との間には有意差が認められた。

これらのことより，高濃度のグリセリンは鶏精子に対して形態学的および生理学的に悪影響を及ぼすものと推定され，およそ7％のグリセリン濃度が鶏精子の凍結保存に対し最適であると考えられた。