Comparison of Cryoprotectants and Methods of Cryopreservation of Fowl Spermatozoa

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ABSTRACT The deleterious effects of three cryoprotectants, glycerol, dimethylsulfoxide (DMSO), and dimethylacetamide (DMA), were compared on fowl spermatozoa. The viability and integrity of spermatozoa were measured with eosin-nigrosin smears. Glycerol was the least deleterious cryoprotectant, followed by DMA, and DMSO was the most toxic. Methods employing either glycerol or DMA were then compared for the cryopreservation of semen in either straws or pellets. Fertility was measured following artificial insemination.

The highest fertility rates were obtained with semen frozen with DMA in pellets directly plunged in liquid nitrogen, DMA being added at –6 C (92.7%) or 5 C (84.7%). When semen was frozen in straws, glycerol equilibrated for 1 or 30 min gave the highest fertility results, but the fertility rates were lower (53.7 and 63.9%) than with DMA in pellets. The lowest results (26.7%) were obtained when semen was frozen in straws with DMA.

When semen was frozen in pellets at very high cooling rates, DMA was superior to glycerol as a cryoprotectant, as evidenced by fertility. In contrast, when straws and low freezing rates were used, glycerol gave better results; however these results were never as high as those obtained with DMA and pellets. In conclusion, under our experimental conditions, the highest fertility rates were achieved with DMA and pellets. However, for gene banking, which requires high levels of safety and clear identification, glycerol and straws are more convenient.

(*Key words*: cryopreservation, spermatozoa, fertility, artificial insemination, fowl)

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INTRODUCTION

Cryopreservation of fowl semen has been studied for more than 50 yr (Schaffner *et al*., 1941) and a variety of procedures have been developed (Lake, 1986; Hammerstedt and Graham, 1992; Bellagamba *et al*., 1993). Each procedure has its own particular variables: diluent, dilution rate, cooling rate, nature of cryoprotectant, rate and type of incorporation of the cryoprotectant, freezing conditions, choice of packaging during freezing, and thawing procedures.

One of the most critical steps in successful cryopreservation of fowl semen is the choice of the cryoprotectant and its use during the process. A variety of cryoprotectants, i.e., glycerol, dimethylsulfoxide (DMSO), and dimethylacetamide (DMA), have been used in the past (Sexton, 1978; Lake *et al*., 1981; Lake and Ravie, 1982, 1984). Any given cryoprotectant can be used to obtain acceptable fertility levels providing that hens are inseminated with adequate numbers of thawed sperm (Schramm, 1991; Seigneurin and Blesbois, 1995; Tselutin *et al*., 1995; Van Voorst and Leenstra, 1995).

Nonetheless, packaging is an important additional variable to consider when trying to optimize a cryopreservation method due to the number of variables involved in any given method and the extent to which they interact (Mortimer *et al*., 1976; Sexton, 1979; Duplaix and Sexton, 1984; Lake and Ravie, 1984; Latorre *et al*., 1988).

The aim of the present study was first to compare the potentially deleterious effects of the three main cryoprotectants (glycerol, DMSO, and DMA) on chicken sperm frozen under standardized conditions. The corresponding methods of the two least deleterious cryoprotectants were then compared, in our routine conditions for glycerol (Seigneurin and Blesbois, 1995) and as close as possible to the original procedure for DMA (Tselutin *et al*., 1995).

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Abbreviation Key: DMA = dimethylacetamide; DMSO = dimethylsulfoxide; INRA = Institut National de la Recherche Agronomique.

MATERIALS AND METHODS

Animals

A total of 48 meat type I 99 roosters along with 288 ISA Brown egg laying hens (Institut de Selection Animale, Chateaubourg, France) were used in this study at the Poultry Research Station, Institut National de la Recherche Agronomique (INRA) in Nouzilly, France. The fowl were housed in individual battery cages. The lighting program was 14 h light:10 h dark. The males received 110 g/d of a diet containing 2,974 kcal ME/kg and 125 g/kg crude protein, and the hens received *ad libitum* access to a diet containing 2,795 kcal ME/kg and 165 g/kg crude protein. The males were 63 wk of age and the females were 30 wk of age at the start of the experiment.

Semen Collection

Semen was routinely collected three times a week by abdominal massage (Burrows and Quinn, 1937) and used without any selection according to quantity or quality parameters of semen production.

Experiment 1

Four milliliters pooled semen was collected in 4 mL Lake diluent (Lake, 1968) and gently mixed. The resulting 8 mL of diluted semen was then equally aliquoted for each treatment and cooled within 20 min at 5 C. Four levels (4, 6, 8, and 11%) of glycerol, DMSO, or DMA were then added and equilibrated for 30 min. Osmotic pressures were estimated with an Fiske osmometer.[2] Vital stained semen smears were prepared after equilibration time. There were eight repetitions of the experiment.

Experiment 2

Fertility Rates Obtained from Semen Frozen in Either Glycerol or DMA. Glycerol and DMA were used in standardized conditions, either as close as possible to the original sperm freezing method or with slight modifications, in order to unify the conditions of comparison of the cryoprotectants. These modifications involved varying the time of equilibration for glycerol (1 or 30 min) and the temperature of the chamber containing the diluted semen before addition of the cryoprotectant (5 or –6 C) for DMA. New thawing equipment compared to the thawing procedure for the original DMA method (Tselutin *et al*., 1995) was used. Combinations of rapid freezing-thawing and pellet packaging or relatively slow freezing-thawing and straw packaging were compared in order to adapt the original DMA method to the use of straws.

Prefreezing Procedures

Five prefreezing procedures were applied to pooled semen (Table 3). Treatment 1: Four milliliters of clean semen were collected in 4 mL Lake diluent (Lake, 1968) at room temperature. Semen and diluent were gently mixed after collection from each male. Diluted semen was cooled to 5 C over 20 min. The diluted and cooled semen was then mixed with 4 mL of the same diluent containing 1.32 mL glycerol, which was previously prepared and cooled to 5 C (final concentration 11%). The diluted semen and cryoprotectant were equilibrated for 30 min at 5 C. Semen was transferred to 0.5 mL I.M.V. plastic straws[3] after equilibration, and the straws were sealed.

Treatment 2: Semen samples were treated as in Treatment 1 except that the equilibration time was reduced to 1 min to be comparable with Treatments 3, 4, and 5.

Treatment 3: A total of 4 mL clean semen was collected in 4 mL diluent. Semen and diluent were gently mixed after collection from each male. Diluted semen was cooled for 20 min at –6 C in a portable refrigerator.[4] After these 20 min, the effective temperature of the diluted semen was 5 C. Up to 6% DMA was then added in bulk and equilibrated for 1 min.

Treatment 4: The semen was treated as in Treatment 3, except for the cooling temperature. The diluted semen was cooled for 20 min at 5 C, as in the glycerol procedure used in Treatments 1 and 2.

Treatment 5: Semen was treated as in Treatment 4 until freezing. After 1 min of equilibration with DMA, semen was transferred into 0.5-mL plastic straws previously stored at 5 C and sealed as in Treatments 1 and 2.

Freezing Procedures

In Treatments 1, 2, and 5, the straws were placed in a biological freezer unit[2] and frozen from 5 to –35 C at 7 C/min and from –35 to –140 C at 20 C/min. The straws were then plunged into liquid nitrogen.

In Treatments 3 and 4 semen samples were dropped (about 0.05 mL each drop) directly in liquid nitrogen and resulted pellets stored in liquid nitrogen.

Thawing Procedures

In Treatments 1, 2, and 5, straws were thawed in a water bath at 5 C. After thawing, the straws were quickly opened and the semen transferred to a glass beaker. In Treatments 1 and 2, the semen was rediluted and glycerol was removed by centrifugation as described by Seigneurin and Blesbois (1995). To avoid the contraceptive effect of the glycerol, the supernatants were then discarded, and the resulting pellets containing spermatozoa were suspended in 100 μL diluent (Lake *et al*., 1981).

In Treatments 3 and 4, the pellets were quickly thawed on thermoregulated hotplates at 60 C. In Treatments 2, 3, and 4, DMA was not removed.

[2]Fiske Associates, Needham Heights, MA 02194.
[3]I.M.V. B.P. 81, 61300 L'Aigle, France.
[4]Engel Sawafugi Electric Co. Ltd., Japan.

Semen Quality Tests

Viability Tests. Sperm viability was determined using the eosin nigrosin test (Lake, 1979; Haije, 1990) A total of 300 spermatozoa were counted per slide with 10 replicates examined per treatment. The results were expressed as the percentage of live and morphologically normal cells.

Fertility Tests. Sperm concentrations were estimated with a presealed photometer at a wavelength of 540 nm. The final concentrations of ready-to-use artificial insemination doses ranged from 2.5 to 3.5×10^9 spermatozoa per milliliter. A total of 48 hens per treatment were inseminated twice a week for a total of five intravaginal inseminations (300 million sperm per hen). Artificial inseminations were performed at 4 cm depth with a glass pipette fixed on an automatic gun.[5]

The fertilizing ability of sperm cells was estimated from eggs collected on Days 2 to 4 after each insemination of frozen-thawed sperm and on Days 2 to 8, for the control group. Fertility rates (fertilized eggs/incubated eggs × 100) were determined by candling eggs on Day 7 of incubation.

Statistical Analyses

The percentages of live and morphologically normal spermatozoa were examined by analysis of variance and the Newman-Keuls test (Dagnelie, 1975). Fertility rates were compared by the Fisher PLSD test after transformation of variables in arc sine square root percentages.

RESULTS

Deleterious Effects of Glycerol, DMA, and DMSO on Fresh Sperm

The proportions of morphologically normal and viable spermatozoa were higher in samples treated with glycerol (72 to 76%) than in those treated with DMA (62 to 68%), whatever the concentration tested (Table 1). The lowest results (22 to 26%) were obtained with DMSO. All these values were significantly lower than those observed before the addition of the cryoprotectant (92% normal and viable spermatozoa). The osmolarity of the diluents increased as the concentrations of cryoprotectants increased (Table 2). There was no concentration effect on viability of sperm cells, whatever the cryoprotectant.

Effects of "Glycerol" and "DMA" Methods on Fertilizing Ability

The highest fertility rates were obtained with undiluted fresh semen (94.7%) (Table 3). However, spermatozoa frozen and thawed in pellets with DMA yielded fertility rates (84.7 to 92.7%) that were not significantly different from those obtained with fresh undiluted semen. The temperature of addition of DMA (5 or –6 C) had no significant effect on the mean results of the five successive artificial insemination. The fertility rates obtained with semen frozen-thawed in straws with either glycerol or DMA were lower (26.7 to 63.9%). However, with straws packaging, glycerol gave far higher fertility rates (53.7 to 63.9%) than DMA (26.7%). The glycerol equilibration time (1 or 30 min) did not significantly affect the results.

TABLE 1. Liver and morphologically normal fowl spermatozoa (eosin-nigrosin) incubated in Lake diluent with the addition of glycerol, DMA, or DMSO[1]

Cryoprotectant level	Glycerol	DMA	DMSO
	(%)		
0	93 ± 0.8[a]	92 ± 1.3[a]	94 ± 0.7[a]
4	76 ± 2.1[b]	68 ± 1.6[cd]	22 ± 1.4[ef]
6	72 ± 2.5[bc]	62 ± 1.9[d]	20 ± 1.7[f]
8	74 ± 2.7[b]	68 ± 2.8[cd]	26 ± 2.6[e]
11	74 ± 2.6[b]	62 ± 2.6[d]	27 ± 2.6[e]

[a–f]Means ± SE with no common superscript differ significantly (n = 8, $P < 0.05$).

[1]DMA = dimethylacetamide; DMSO = dimethylsulfoxide.

DISCUSSION

Various methods of freezing sperm of domestic fowl have been used quite successfully during the last 30 yr (Sexton, 1976; Lake and Stewart, 1978; Seigneurin and Blesbois, 1995; Tselutin *et al*., 1995; Van Voorst and Leenstra, 1995); however all these methods have been used independently and very few attempts have been made to compare them (Haije, 1990; Seigneurin and Blesbois, 1994).

This standardized *in vitro* comparison of the three cryoprotectants, glycerol, DMA, and DMSO showed that DMSO is the most toxic cryoprotectant and glycerol the least deleterious; However, the highest fertility rates were obtained with DMA, but only when spermatozoa were frozen in pellets.

As expected, glycerol, which is a physiological molecule, was less deleterious to sperm cells than DMA and DMSO, which are not physiological molecules of sperm cells. However, the deleterious effects of the

TABLE 2. Osmotic pressures of freezing diluents with the addition of glycerol, DMA or DMSO[1]

Cryoprotectant level	Glycerol	DMA	DMSO
(%)		(mosm)	
4	831 ± 39	870 ± 42	1,033 ± 43
6	1,089 ± 39	1,178 ± 27	1,457 ± 51
8	1,357 ± 142	1,474 ± 45	1,854 ± 35
11	1,664 ± 161	1,902 ± 118	3,362 ± 60

[1]DMA = dimethylacetamide; DMSO = dimethylsulfoxide.

[5]J. C. Diffision, BP 10, 61550 La Ferté-Fresnel, France.

TABLE 3. Individual and mean ± SE fertility rates obtained from fowl semen frozen with glycerol or dimethylacetamide (DMA) in diluents submitted to various temperature, equilibration, and packaging conditions

Treatment	Cryoprotectant	Temperature of chamber during cooling	Time of equilibration	Packaging	Fertility (n eggs) Successive artificial inseminations					Mean fertility (n eggs)
		(C)	(min)		1	2	3	4	5	(%)
1	Glycerol	5	30	Straws	67.4 (132)	62.9 (132)	57.4 (127)	62.3 (130)	67.9 (131)	63.9 ± 1.9[b] (652)
2		5	1	Straws	36.2 (138)	62.3 (130)	56.8 (125)	61.4 (135)	52.5 (139)	53.7 ± 4.7[b] (667)
3	DMA	–6	1	Pellets	73.7 (118)	88.5 (131)	92.4 (133)	94 (134)	97 (136)	92.7 ± 4.1[a] (630)
4		5	1	Pellets	78.9 (109)	81.2 (133)	83 (124)	87 (131)	92.4 (132)	84.7 ± 5.3[a] (629)
5		5	1	Straws	25.9 (135)	42.5 (134)	27.5 (127)	22.3 (130)	15.4 (136)	26.7 ± 4.5[c] (662)
Control	Fresh undiluted semen				94 (167)		94.9 (197)		95.5 (112)	94.7 ± 0.4[a] (476)

[a–c]Means with no common superscript differ significantly ($P < 0.05$).

cryoprotectants were not dependent on their concentration in this study (from 4 to 11%), although the osmotic pressure of the media increases regularly with the concentration. This result means that the hypertonicity of the media and the osmotic effect originating from the entry of the cryoprotectant into the cells are not the only factors responsible for the deleterious effect of these cryoprotectants.

The predominant *in vitro* "toxic" effect of DMA on spermatozoa compared to that of glycerol was confirmed by fertility tests on semen packaged in straws. When used under conditions similar to the classical methods (slow cooling rate, straws) spermatozoa treated with glycerol provided higher fertility results than spermatozoa treated with DMA, despite the fact that the post-thawing centrifugation of glycerol-treated samples provides an additional deleterious step for sperm (Seigneurin and Blesbois 1994); which does not exist with DMA.

Another potential source of variability between the two techniques comes from the different composition of the diluents used, although both contain the same quantity of polyvinyl pirrolidone (3 g/L).

Finally, it appears that a major source of variation in the overall fertility results observed from glycerol- or DMA-based samples resulted from the combination of two factors: the rapidity of freezing and the type of packaging (pellets or straws). Fertility results were thus far higher with rapid freezing-thawing rates and pellets packaging than with slower, controlled freezing-thawing rates and straw packaging. Fertility rates obtained with the modified method of Tselutin *et al.* (1995) were even higher than those initially reported. These rates might have resulted from the use of hotplates for thawing, thus ensuring faster thawing rates than the original water bath. Because of the nature of the plastic straws, it is, in fact, impossible to freeze and thaw semen in straws as fast as in pellets.

The very rapid freezing rates raise questions concerning the state of the sperm cells after very rapid freezing. Are they really in a frozen crystallized state or are they closer to the vitrification state?

Although the present results are encouraging, especially with semen of unselected meat type males, they suggest that new types of plastic straws resistant to very rapid freezing rates are necessary for further DMA use in identifiable packaging systems. Indeed, straw packaging is more efficient for safety reasons and for convenient identification of the ejaculates, especially for applications in the field of gene banks and breeding. Such studies are in progress in our laboratory.

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